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Presence of corticotropin in brain of normal and hypophysectomized rats

(α-melanotropin/hypothalamus/pituitary)

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ABSTRACT **Immunoreactive and bioreactive corticotropin (ACTH-like) activities have been detected in the median eminence and remaining medial basal hypothalamus of both normal and hypophysectomized adult male rats: bioreactive ACTH (pg/100 μg of protein) 1028 in median eminence and 1289 in medial basal hypothalamus; immunoreactive ACTH (midportion ACTH antibody), 1554 in median eminence and 1887 in medial basal hypothalamus. By use of appropriate antibodies and bioassay, it was demonstrated that immunoreactivity was not due solely to α-melanotropin, which has previously been reported to be present in the brain of hypophysectomized animals. The Sephadex G-50 gel filtration patterns determined by immunoassay of column eluates obtained from hypothalamic extracts of normal or hypophysectomized animals were similar but were not identical to the pattern derived from whole pituitary. Immunoreactive (midportion ACTH antibody) ACTH concentrations (pg/100 μg of protein) of other central nervous system areas in normal animals were: cerebellum 34.3, cortex 46.3, thalamus 23.8, and hippocampus 116.3.**

The total amount of bioreactive ACTH present in the median eminence and medial basal hypothalamus is approximately 1% of that present in the pituitary. The present data suggest that such ACTH may have a diencephalic rather than pituitary origin and raise the question of the functional significance of such ACTH.

The presence of material with bioassayable corticotropin (ACTH) and melanotropin (MSH) activities, with elution volumes on carboxymethylcellulose chromatography similar to those of $ACTH^{1-39}$ and α-MSH and β-MSH, has been reported in hog (1) and dog (2) hypothalamus uncontaminated with pituitary tissue. ACTH and ACTH fragments have been reported to play an important role in motivation, learning, and memory (3). Additionally, $ACTH^{1-28}$, $ACTH^{4-10}$, and $ACTH^{11-24}$ have been demonstrated to have affinity for opiate receptor sites in rat brain *in vitro* (4). There is also recent evidence that the endogenous brain substances that act as agonists at opiate receptor sites, termed "enkephalins" (5) or "endorphins" (6), are COOH-terminal fragments (61–91) of β-lipotropin. Brain extracts incubated with β-lipotropin generate fragments with morphinomimetic activity (7). Endorphin-like activity has also been identified in the pituitary gland (8). Structural relationships are evident between β-lipotropin and ACTH as well as α-MSH and β-MSH (Fig. 1).

The origin of the ACTH-like activity found in the hypothalamus and the mode of entry whereby it affects central nervous system function are unclear. It has been suggested that ACTH or fragments of it may enter the brain via discharge from the anterior pituitary into the cerebrospinal fluid, by retrograde vascular transport along the pituitary stalk, or via the systemic vascular system (9), although systemic injection of radioactively labeled human $\alpha ACTH^{1-39}$ ($\alpha hACTH^{1-39}$) results in insignificant amounts of label appearing in brain (ref. 10; D. Puett, personal communication). It has also been suggested (1) that peptides related to ACTH may have a diencephalic origin. In support of this last suggestion, we now report the presence of immunoassayable and bioassayable ACTH in the median eminence and medial basal hypothalamus of normal rats and persistence of such ACTH in these areas of hypophysectomized rats.

Abbreviations: ACTH, corticotropin; MSH, melanotropin; hACTH, human ACTH; pACTH, porcine ACTH.

MATERIALS AND METHODS

The median eminence and medial basal hypothalamus were removed from 16 control and 16 hypophysectomized (10 days postoperatively) adult male Sprague-Dawley rats as previously described (11), as were the other, larger, regions such as the cerebellum, cerebral cortex, thalamus, and hippocampus. The median eminence and medial basal hypothalamus were dissected from frozen sections of the brain; the other areas of the brain were removed from fresh tissue. The medial basal hypothalamus sample consisted of three 3-mm × 3-mm triangles of brain, each one 0.3 mm thick.

Hypophysectomy was confirmed by visual inspection of the sellar area at time of sacrifice; additionally, plasma ACTH‡ concentrations at this time (between 0900 and 1000 hr) were less than 15 pg/ml in all the hypophysectomized animals and ranged from 27 to 153 pg/ml in the control group (1 ml is equivalent to approximately 55 μg of protein). Specimens of cortex, hippocampus, thalamus, and cerebellum were also obtained from normal animals ($n = 5$). All tissues were analyzed in pools comprised of samples from five or six animals.

Immediately after removal, tissues were placed in plastic or heavily silicone-treated microhomogenizing tubes kept on ice, and were homogenized in 0.1 M HCl (0.2–0.5 ml/mg wet

‡ Immunoassay (12) was performed on unextracted plasma by using the paradoxical binding phenomenon described by Matsukara *et al.* (13) and an antibody raised in our laboratory. On a molar basis, the antibody crossreacted equally with porcine ACTH (pACTH), hACTH, and $ACTH^{1-24}$; α-MSH had approximately 8% crossreactivity. Over the effective range of the standard curve, $ACTH^{1-10}$, $ACTH^{17-39}$ (synthetic human sequence), and β-MSH were undetectable. At a titer of 1/1500, the useful standard dose range was 0–150 pg/ml, with 2–4 pg/ml routinely being significantly different than the zero dose. $pACTH^{1-39}$ was used as a standard and for iodination. Most plasma specimens exhibited parallelism with the standard curve, with acceptable incubation damage to label at plasma concentrations of up to 20% of incubation volume. Plasma was assayed at dilutions of 2–10%. All specimens were assayed in duplicate at multiple dilutions. Ten pools of rat plasma (range, 18–300 pg/ml) were compared in assays with the "paradoxical" antibody and the NH_2-terminal antibody provided by the National Institutes of Health (extracted plasma). Levels obtained with the latter averaged 84% of those with the paradoxical antibody, with an average coefficient of variation of 9.7% ($r = 0.99$; slope = 1.1).

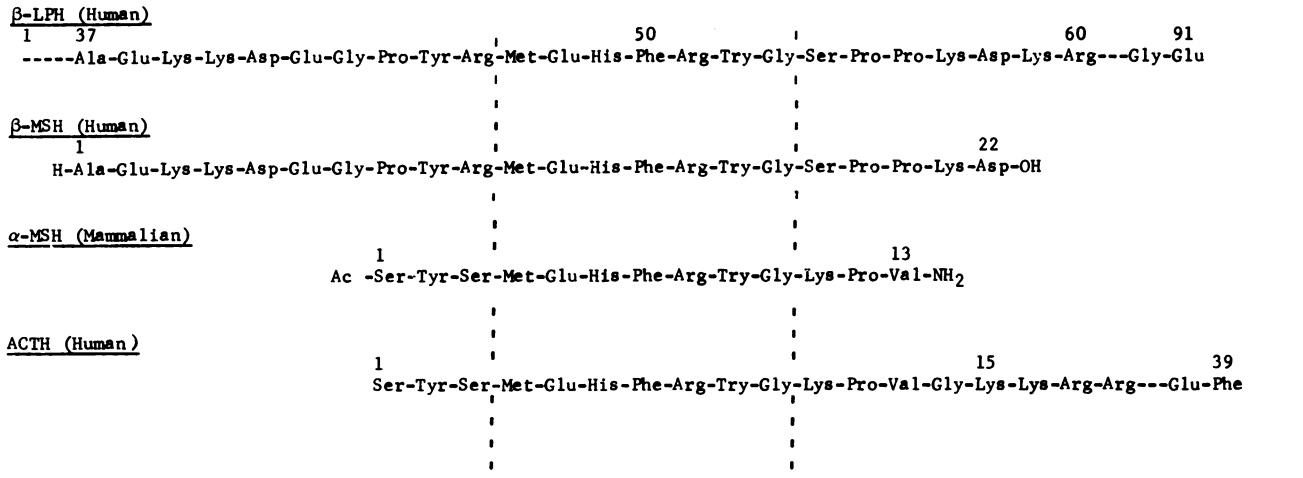

FIG. 1. Amino acid sequences of β-lipotropin (β-LPH), α-MSH, β-MSH, and ACTH. The sequence between the vertical broken lines is shared by all.

weight) to inactive proteolytic enzymes; 2–5 μl of the uncentrifuged homogenate was removed for protein determination. A volume (equivalent to the initial volume of acid) of 80% acetone in 0.1 M HCl was then slowly added, and the tissue was rehomogenized, and centrifuged at 6000 × *g* for 10 min at 4°. The supernatant was decanted, and the tissue pellet was resuspended in 40% acetone in 0.1 M HCl and rehomogenized. (No ACTH is detectable in tissue residue after such extraction; this is not the case when tissue is homogenized in HCl alone.) The supernatants were combined, and these acetone extracts were dried, in a 45° water bath, with a gentle stream of N_2.

The residue was redissolved with a small volume of 0.01 M HCl (100 μl/500 μg of original protein content). This solution was then appropriately diluted with 0.05 M phosphate buffer, pH 7.8, containing 0.25% human serum albumin for the ACTH immunoassay (12) studies utilizing both the Kendall (NH_2-terminal) and West (midportion) antibodies[§] supplied by the NIAMDD Hormone Distribution Program (National Pituitary Agency). They were diluted with Earle's balanced salt solution, pH 7.2–7.4, containing 0.5% bovine serum albumin for ACTH bioassay (12) studies. There is no measurable immunoreactive or bioreactive ACTH-like material in human or bovine serum albumin. Diluted extracts were added directly to the assay system. These extracts exhibited parallelism with reference standard, as evidenced by a highly significant value for the slope of the common regression and an insignificant deviation of reference standard and unknown from this regression line as determined by analysis of variance.

The nature of the ACTH present in pools of 16 medial basal hypothalami from normal and hypophysectomized animals and in 18 individual whole pituitary glands was further characterized by chromatography on Sephadex G-50 (fine). Column dimensions were 1 × 25 cm; 200-μl sample volumes were applied. Columns were equilibrated and eluted with 0.5% human serum albumin (heat-inactivated) in 0.9% saline at room temperature (20–22°); 1-ml fractions were collected. Markers were bromphenol blue-human serum albumin for V_0, ^{125}I-labeled $pACTH^{1-39}$ for ACTH, and $^{125}I^-$ for the salt peak. In this system, unlabeled $ACTH^{1-39}$ migrates with the same filtration characteristics as ^{125}I-labeled $pACTH^{1-39}$.

[§] The NH_2-terminal antibody reacts almost equally on a molar basis with $ACTH^{1-39}$, $ACTH^{1-24}$, and α-MSH but not with $ACTH^{17-39}$, $ACTH^{11-24}$, or $ACTH^{1-10}$; the midportion antibody reacts with $ACTH^{1-39}$ and $ACTH^{11-24}$ on an equimolar basis, but not with α-MSH, β-MSH, $ACTH^{1-10}$, or $ACTH^{17-39}$.

RESULTS

The concentration of ACTH in the median eminence, as measured by either immunoassay or bioassay, was similar to that in the medial basal hypothalamus (Fig. 2). Immunoreactive and bioreactive ACTH content in the medial basal hypothalamus was approximately 10-fold greater than that in the median eminence (NH_2-terminal antibody: median eminence content 1400 pg, medial basal hypothalamus content 14,419 pg; midportion antibody: median eminence content 571 pg, medial basal hypothalamus content 6057 pg; bioassay: median eminence content 388 pg, medial basal hypothalamus content 4138 pg). The only significant difference in ACTH concentrations between tissues from control and hypophysectomized animals was in the case of median eminence—ACTH concentrations determined with the midportion antibody were lower in the hypophysectomized group. The ratios of immunoassayable to bioassayable ACTH for control animals was 1.51 in median eminence and 1.46 in medial basal hypothalamus with midportion antibody and 4.37 and 3.44, respectively, with NH_2-terminal antibody. The ratios in hypophysectomized animals were lower with the midportion antibody—1.27 in median eminence and 1.34 in medial basal hypothalamus—whereas with the NH_2-terminal antibody these ratios were 3.94 in median eminence and 4.38 in medial basal hypothalamus, ACTH concentrations (pg/100 μg protein) in normal cerebellum, cortex, thalamus, and hippocampus were 3.43, 46.3, 23.8, and 116.3 (midportion antibody).

The Sephadex G-50 gel filtration patterns of medial basal hypothalamus pools derived from control and hypophysectomized animals are depicted in Fig. 3. With a given antibody, similar elution patterns were obtained for both animal groups. Higher values were obtained with the NH_2-terminal antibody for the fractions corresponding to fragments smaller than $ACTH^{1-39}$; these may represent α-MSH (which crossreacts with this antibody but not with the midportion antibody). For comparison, two representative gel filtration patterns of whole rat pituitary are depicted in Fig. 4. Essentially identical patterns were derived for a given antibody. Again, values obtained with the NH_2-terminal antibody were greater for the fractions corresponding to fragments smaller than $ACTH^{1-39}$.

The elution pattern from Sephadex G-50 gel filtration was arbitrarily divided into three sections (to represent activity of the three peaks present: fractions 8–11, 12–16, and 17–22) and the percentage of the total immunoreactivity (midportion antibody, this being more representative of biological ACTH

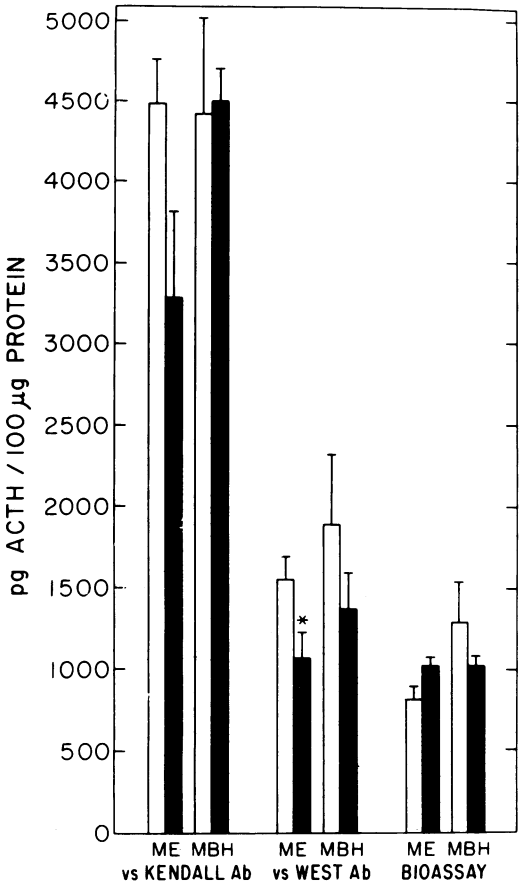

FIG. 2. ACTH-like activity of median eminence (ME) and medial basal hypothalamus (MBH) as determined by assay with two different antibodies (see [8]footnote) and by bioassay (means ±SEM). Open bars indicate control tissue; black bars indicate tissue taken from rats 10 days posthypophysectomy. * = $P < 0.01$ for effect of hypophysectomy.

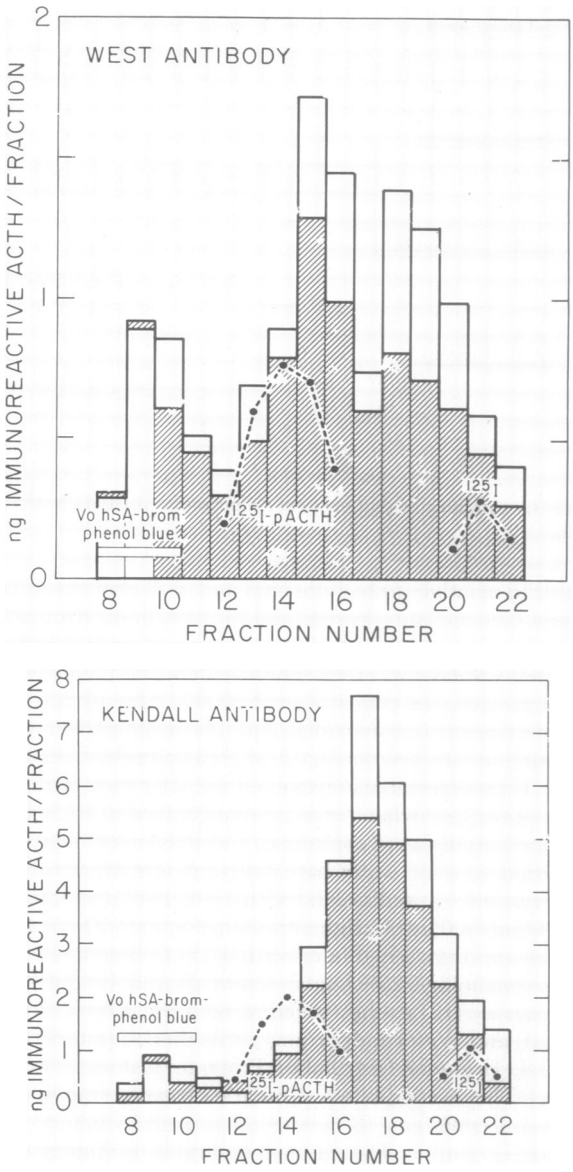

FIG. 3. Sephadex G-50 gel filtration of pooled medial basal hypothalamus extract ($n = 16$). Open bars indicate control tissue; shaded bars indicate tissue taken from rats 10 days posthypophysectomy. *Upper.* Immunoreactive concentrations derived with West (midportion) antibody. *Lower.* Immunoreactive concentrations derived with Kendall (NH_2-terminal) antibody. Note different ordinate scale. With this antibody, a greater portion of immunoreactivity is seen in fractions 16–22 than with the West antibody.

activity) that each of these divisions represented was calculated. No differences were seen between such percentages from pituitary compared with control or hypophysectomized medial basal hypothalamus (Table 1). From Figs. 3 upper and 4 upper, the second and third peaks of the medial basal hypothalamus chromatograms seem to be retarded compared to corresponding peaks in the pituitary chromatograms. The central peak of the pituitary chromatogram was eluted just before the $ACTH^{1-39}$ marker, but in the medial basal hypothalamus chromatogram the central peak was eluted just after the $ACTH^{1-39}$ marker. It should also be noted that only about 40% of the bioreactivity present in the medial basal hypothalamus can be accounted for solely by the central peak ($ACTH^{1-39}$ marker). This would indicate that other areas exhibiting immunoreactivity have some intrinsic bioreactivity.

DISCUSSION

The present data indicate the presence, in extracts of median eminence and medial basal hypothalamus from normal and hypophysectomized rats, of material that is immunoreactive against two antibodies directed against different portions of the ACTH molecule and that has biological ACTH activity in the dispersed adrenal cell assay (12). Additionally, gel filtration yielded evidence of lesser amounts of fragments corresponding to "big" ACTH (14) and to fragments smaller than $ACTH^{1-39}$. These latter fragments may correspond in part to α-MSH-like activity, in view of the differences in immunoreactive concentrations found with the NH_2-terminal and midportion antibodies.[¶] Fragments other than α-MSH, however, must be present in the tissue assayed, in view of the presence of immunoreactive (midportion antibody) ACTH activity in Sephadex G-50 eluates appearing subsequent to the elution of $ACTH^{1-39}$.

Immunoreactive (15) and bioreactive (16) α-MSH has also been reported to be present in rat brain after hypophysectomy,

[¶] Preliminary experiments indicate good correlation between α-MSH-like concentrations derived by immunoassay (NH_2-terminal antibody) after immunoprecipitation with an antibody directed against the midportion of the ACTH molecule and those derived by extrapolation [subtracting immunoreactive ACTH concentrations (midportion antibody) from those measured with the NH_2-terminal antibody].

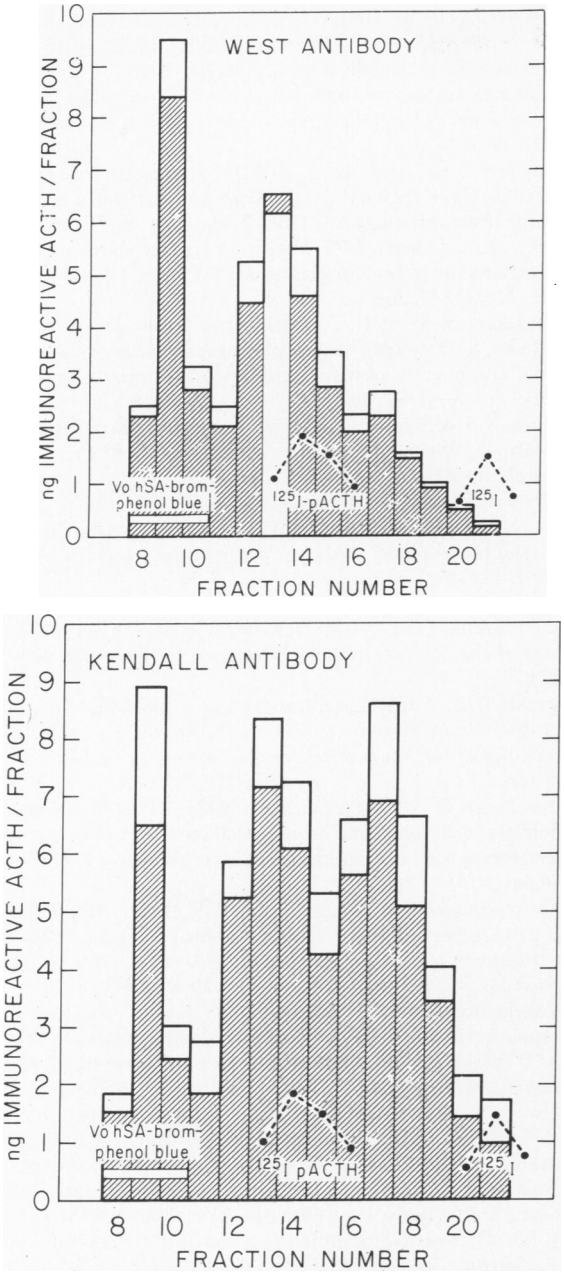

FIG. 4. Sephadex G-50 gel filtration of two normal whole rat pituitaries. Open and shaded bars indicate the two individual specimens. *Upper.* Immunoreactive concentrations derived with West (midportion) antibody. Close agreement is seen in the patterns for the two specimens. *Lower.* Immunoreactive concentrations derived with Kendall (NH_2-terminal) antibody.

although these reports do not state whether such concentrations were similar to those found in intact animals. Immunofluorescence studies (17) suggest that such α-MSH may not be completely identical to authentic α-MSH. In these latter studies it was stated that extrapituitary localization could not be demonstrated with antibodies directed against $ACTH^{1-24}$, but it was not specified which extrapituitary areas were studied.

A question arises as to the origin of the ACTH-like activity present in the hypothalamus. Its presence in the brains of normal animals in the present study (by virtue of the method of dissection used) would not appear to be secondary to contamination with hypophyseal tissue. The presence of such activity in hypothalami 10 days after hypophysectomy also contradicts the possibility of a pituitary source. The possibility that such activity was secondary to inclusion of some elements of pars tuberalis in the extracts assayed is unlikely with the method of dissection used to obtain the areas assayed. The similarity of ACTH concentrations in median eminence and medial basal hypothalamus, the latter of which is remote from any pars tuberalis tissue, also would negate such a possibility. Lastly, the gel filtration pattern obtained from hypothalamic extracts is not identical to that derived from whole pituitary.

Table 1. Distribution of immunoreactive ACTH (mid-portion antibody) in different fractions obtained on Sephadex G-50 (fine) chromatography

Sample	Distribution (%) A*	B*	C*
Control whole pituitary†	19–46	33–47	18–26
Medial basal hypothalamus (MBH)‡			
Control	22	42	36
Hypophysectomized	26	43	30

* Fractions defined by V_e/V_0: A = 1.0 (pituitary, MBH); B = 1.44 (pituitary), 1.66 (MBH); C = 1.88 (pituitary), 2.0 (MBH); $ACTH^{1-39}$ = 1.55; salt peak = 2.44.
† n = 18.
‡ Pool obtained from 16 animals.

One possible explanation for the presence of ACTH-like activity in brain would be the existence of ACTH receptors, which might theoretically prolong the half-life of the bound ACTH molecule, although calculations based on the dissociation constant of ACTH bound to adrenal receptors make it unlikely that detectable ACTH would be present 10 days after hypophysectomy. There have been reports that the presence of neurosecretory axons can initiate the morphological and functional development of adenohypophysial cell types from nonadenohypophyseal tissues (18). Evidence of maintained adrenocortical activity has been claimed in hypophysectomized dogs bearing a transplant of salivary gland in the place of the extirpated hypophysis (19).

Another possibility, as originally suggested by Guillemin *et al.* (1), is that the ACTH activity seen in the brain extracts arises from neural sources. The present demonstration that such activity is present in the brains of hypophysectomized animals further strengthens this suggestion.

Because of differences in anatomical extent of areas assayed, methods of tissue extraction, use of different species, and use of antibodies with different characteristics, it is difficult to make valid comparisons between the ACTH-like activity reported in the present study and that reported by others (20, 21) for either median eminence or NIH-HME-RP reference extract. Immunoreactive (midportion antibody) and bioreactive ACTH concentrations of median eminence and medial basal hypothalamus observed in the present study are approximately 1/25th and 1/30th respectively, of those observed by us and others (22) for whole pituitary. Posterior pituitary concentrations of bioreactive ACTH have been reported (22, 23) to be one-fourth of those found in the anterior lobe; most of the ACTH content of the posterior pituitary (which includes both pars intermedia and posterior lobe) is material with COOH-terminal-like immunoactivity (COOH-terminal/bioreactive ratio, approximately 100:1) (22). Since the weight of median eminence plus medial basal hypothalamus is about one-third that of the pituitary, the total amount of bioreactive ACTH in this area is roughly 1/100th that in pituitary.

Lesser concentrations of immunoreactive ACTH (midportion antibody) were present in other central nervous system areas

in normal animals. In view of the greater total weight of these areas, ACTH present there may constitute a significant portion of the ACTH found in brain. The biological nature of such ACTH remains to be demonstrated, although the present studies show good correlation between immunoreactive concentration (midportion antibody) and bioreactive ACTH concentration. It is tempting to speculate about the biological function of such ACTH. Preliminary observations indicate significant depression ($P < 0.01$) of immunoreactive (midportion antibody) ACTH content of median eminence but not of medial basal hypothalamus after administration of 1500 μg of corticosterone daily or after 3 days of treatment with a long-acting ACTH preparation (Synacthen Depot). More significant would be the demonstration of changes in median eminence and medial basal hypothalamus ACTH content after "neural" stresses. Because plasma ACTH is undetectable and adrenal weight and adrenal function are not maintained in hypophysectomized rats, it may well be that ACTH present in brain has extrapituitary actions, possibly in learning, memory, and motivation as suggested by DeWied (3).

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