Effect of Hypothalamic Extracts on Protein Synthesis in Rat Anterior Pituitary Tissue* (33037)

C. K. GRIESHABER AND W. C. HYMER (Introduced by Adam Anthony)

Physiology Section, Department of Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

It has been known for several years that slices of rat anterior pituitary tissue rapidly incorporate labeled amino acids into protein (1). Recently it has been shown that labeled amino acids are incorporated into ACTH in pituitary organ culture (2); into prolactin and growth hormone in bovine pituitary slices (3); and into ACTH in cell-free systems containing pituitary ribosomes (4). Furthermore, Kracier (5) and Bloemendal *et al.* (6) have demonstrated the presence of polyribosomes in pituitary tissue and have suggested that hormone synthesis occurs on these subcellular particles.

Acid extracts of rat hypothalamus are known to initiate release of biologically detectable quantities of TSH[1] (7, 8), FSH (9), and STH (10) from rat anterior pituitary *in vitro*. These extracts may also initiate *synthesis* of new protein hormone (7, 10–12), but their direct effect at the cellular level has not, as yet, been clearly demonstrated. The results in the present study suggest that hypothalamic extracts stimulate protein synthesis in pituitary explants, and indicate that this effect occurs at the polyribosome level.

Materials and Methods. Male Holtzman rats (Madison, Wisconsin) ranging in weight from 180–220 gm were maintained on a 12-hour light, 12-hour dark cycle with food and water *ad libitum*. Animals were killed by cervical dislocation, pituitary glands were removed and posterior lobes were discarded.

Incubation conditions and preparation of fresh acid extracts of hypothalamic tissue were essentially the same as those reported by Sinha and Meites (7). Amino acid-^{14}C (UL) mixture (> 180 mC/mmole) or glutamic-^{14}C (UL) acid (~1 mC/mg) (New England Nuclear Corp.) was added to the incubation medium (Medium 199, Difco Labs.) to a concentration of 2μC/ml. The pituitaries were incubated in 10-ml Erlenmeyer flasks (1.5 ml of medium/gland) in a Dubnoff shaker at 60 cycles/min and 37°C under a 95% O_2–5% CO_2 atmosphere. Duration of incubation and time at which hypothalamic or cortical extracts (1 equivalent/pituitary) were added are given in the following section.

Proteins were extracted from pituitary homogenates by a modified Schneider procedure (13). The TCA insoluble fraction was washed four times with 5% TCA and twice with chloroform–ethanol–ether (1:2:1) following hydrolysis in 5% TCA at 90° for 30 min. Protein hydrolysis was carried out in 0.1 *N* NaOH overnight at room temperature. Protein content was estimated by a modified Lowry (14) procedure using BSA as standard. Radioactivity was assayed in a Nuclear Chicago liquid scintillation spectrometer using Bray's solution (15). Counts were corrected to a uniform efficiency using hexadecane-^{14}C as standard. When glutamic acid-^{14}C was used, the homogenizing medium and TCA contained 10 m*M* glutamic acid-^{12}C.

After incubation in the amino acid-^{14}C mixture, polyribosomes were prepared from pituitary glands by a modification of the method of Munro *et al.* (16). The glands were washed with cold medium A (50 m*M* Tris buffer, pH 7.5; 25 m*M* KCl; 5 m*M* $MgCl_2$; and 250 m*M* sucrose), pooled and homogenized in medium A. Intact cells, nuclei, and mitochondria were removed by cen-

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[1] Abbreviations: TSH, thyroid stimulating hormone; FSH, follicle stimulating hormone; STH, somatotropic hormone; ACTH, adrenocorticotropic hormone; TCA, trichloroacetic acid; DOC, sodium deoxycholate; HE, hypothalamic extract; CE, cortical extract; BSA, bovine serum albumin.

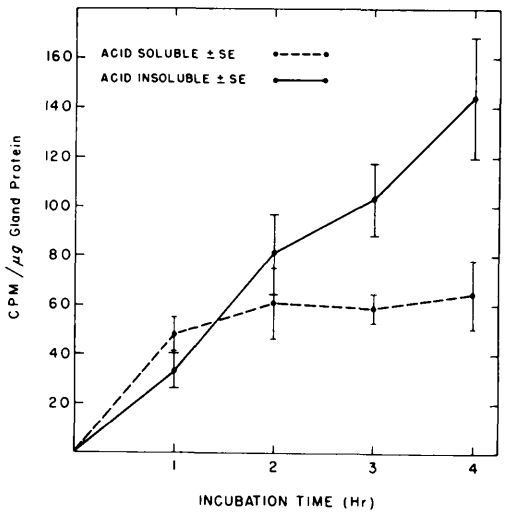

FIG. 1. Rate of uptake [acid soluble (---)] and incorporation [acid insoluble (—)] of amino acid-^{14}C mixture into protein of rat anterior pituitary glands *in vitro*. Each flask contained the equivalent of 1 gland in halves. Individual points represent the average of 4 experiments ± SE.

trifugation at 10,000*g* for 5 min.

The postmitochondrial supernatant was centrifuged at 105,000*g* for 2 hours in the SW-39 rotor of a Spinco model L ultracentrifuge and the microsomes were resuspended in buffer B (10 m*M* Tris buffer, pH 7.4; 10 m*M* KCl; and 1.5 m*M* $MgCl_2$). The DOC was added to a final concentration of 1%. After 5 min at 0–4°C the suspension was layered onto a precooled 10–30% (w/w) linear sucrose gradient prepared in buffer B. Following centrifugation at 105,000*g* for 60 min, 0.3-ml fractions were collected by tube puncture. In one experiment, an aliquot of the DOC treated suspension was incubated with ribonuclease for 5 min at 37°C. In some cases polysomes were isolated directly from a DOC treated postmitochondrial supernatant (see below).

Fractions were diluted to 2 ml with buffer B and absorbance was measured at 235, 260, and 280 mμ in a Beckman DU spectrophotometer. The BSA (200 μg) was added to each fraction and the proteins extracted as described above.

Results and Discussion. The rate of incorporation of amino acid-^{14}C mixture into the protein fraction of anterior pituitary gland halves incubated *in vitro* is shown in Fig. 1. Synthesis, as reflected by the incorporation of labeled amino acids into an acid insoluble product, was essentially linear for 4 hours (the longest incubation period thus far studied). Approximately 75–80% of the 40,000–50,000 counts in the glands was in acid precipitable form after 4 hours of incubation. These findings are consistent with those of others (1, 2, 16, 17) and indicate the rapid rate of protein synthesis occurring in this system.

In some experiments, pituitary polyribosomes have been isolated from glands following *in vitro* incubation. A typical polysome profile obtained from 8 pituitaries after a 30-min pulse with ^{14}C labeled amino acids is shown in Fig. 2A. The distribution of radioactivity in the gradient indicates that protein synthesis is occurring on ribosomal aggregates. As shown in Fig. 2B, the labeled material in the heavy regions of the gradient is extremely sensitive to ribonuclease; approximately 64% of the counts in fractions 1–9 appear in the monomeric region (fractions 10–11) following mild ribonuclease treatment. The small ribonuclease resistant peak (fraction 7) is of interest and may represent an artifact in the preparation of the polysomes as has been recently suggested by Humphreys and Bell (18).

The effects of freshly prepared acid extracts of hypothalamic (HE) and cerebral cortices (CE) on the rate of protein synthesis in anterior pituitary glands in organ culture are shown in Fig 3. Glutamic acid-^{14}C was used in these experiments since this amino acid is relatively common in most anterior pituitary protein hormones (19, 20). The mean specific activity (cpm gland/μg of gland protein) [Fig. 3(2)] of the HE-treated pituitaries was not significantly different from that of the CE-treated glands for periods up to 2 hours after extract addition. However, when the acid insoluble counts in the medium were included in the calculation of the specific activity (gland cpm + medium cpm/μg of gland protein) [Fig. 3(1)], a significant increase was found in the HE-treated glands. These differences are significant at $p \leq 0.10$, 0.01, and 0.05 at 30,

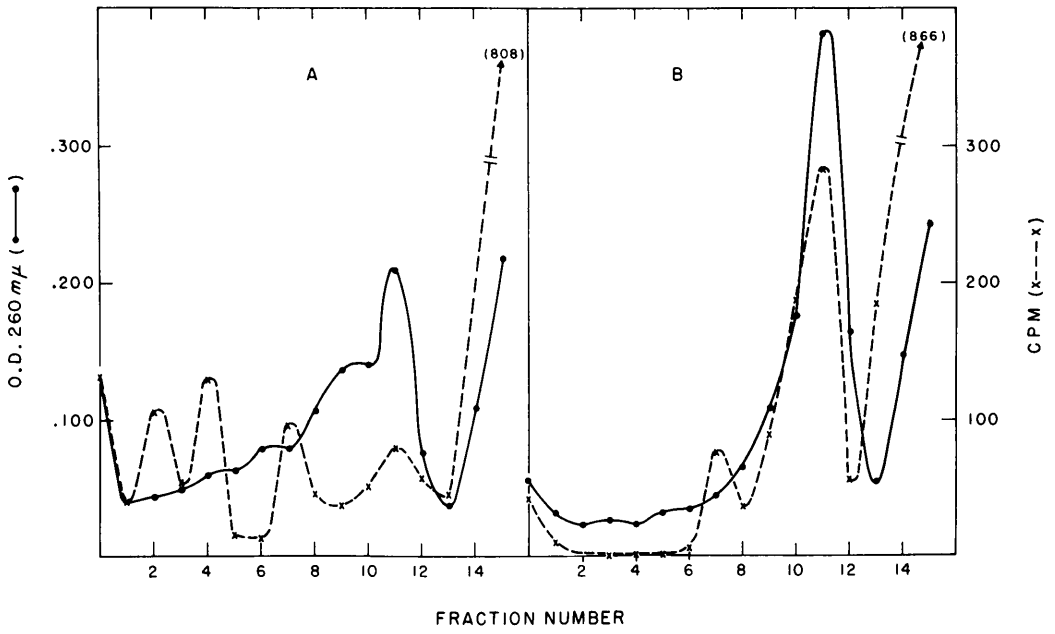

FIG. 2. Sedimentation profile of pituitary polyribosomes. Sixteen anterior pituitary glands were incubated for 30 min in amino acid -^{14}C mixture. One half of the DOC-treated microsomal fraction (see "Methods") was layered directly on a sucrose gradient (A). The other half was incubated in ribonuclease (1 μg/ml, 37°C) 5 min prior to density gradient centrifugation (B). OD_{260} (●); cpm (×).

60, and 90 min of incubation after addition of the extracts. Presumably the difference between the specific activities of HE- and CE-treated glands may be attributed to release of newly synthesized protein from pituitary glands. This suggests that intracellular packaging of newly synthesized hormones into discrete populations of cytoplasmic granules may not be prerequisite to their release.

Importantly, when HE and CE were incubated in glutamic acid-^{14}C in the *absence* of pituitary tissue, radioactivity in the acid insoluble fractions was the same for equivalent amounts of CE and HE. As a maximum estimate, these counts could contribute a specific activity of no more than 1.7 for both of the extracts and thus do not eliminate the validity of the HE effect. In separate experiments

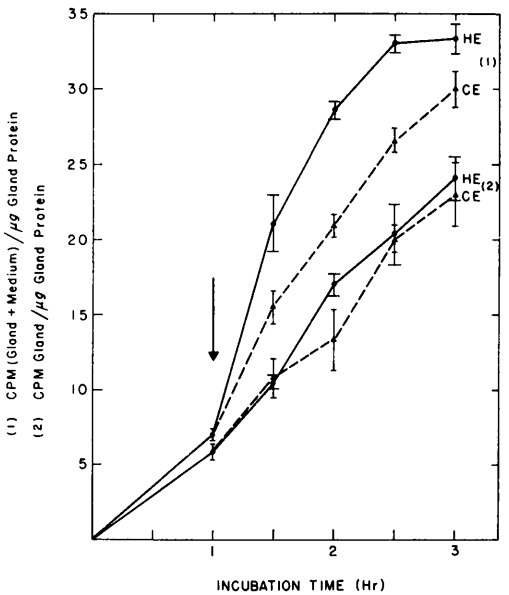

FIG. 3. Effect of HE and CE on incorporation of glutamic acid-^{14}C into protein by anterior pituitary glands *in vitro*. Freshly prepared extracts were added (arrow) to flasks containing 1 pituitary in halves following a preincubation period of 1 hour in labeled medium. In (1), protein counts in the medium are added to the protein counts in the gland and the total counts divided by μg of protein in the gland. In (2) the protein counts in the gland are divided by μg of protein in the gland. Each point represents the average of 3 experiments ± SE.

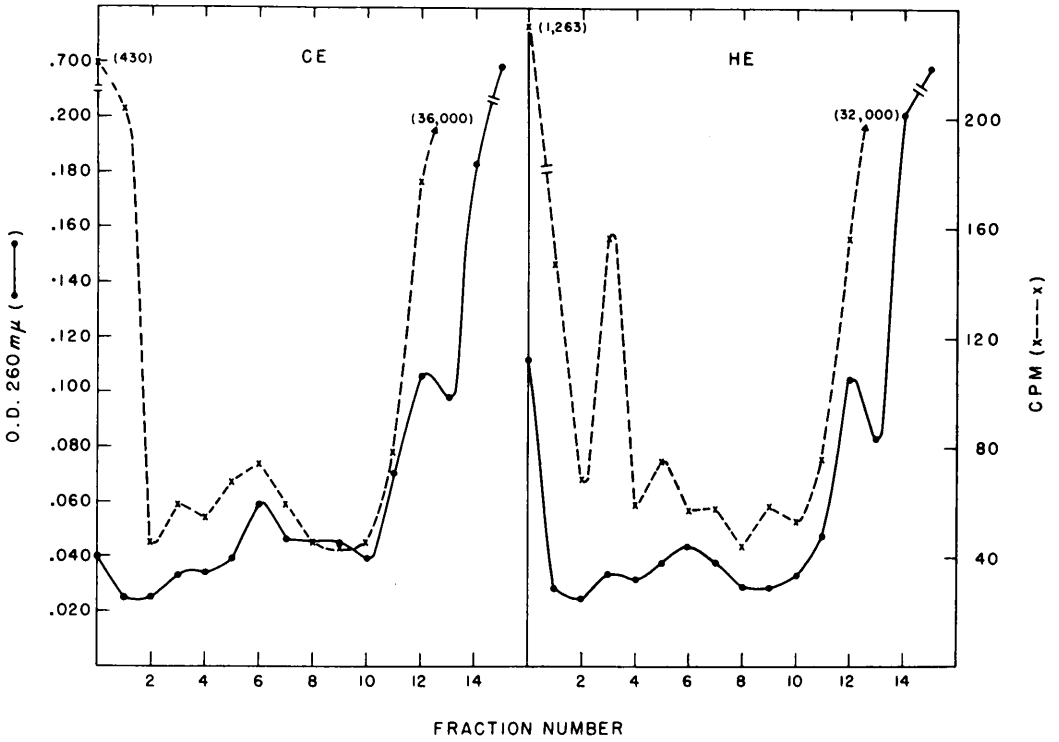

FIG. 4. Effect of HE and CE on pituitary polyribosomes. Sixteen glands were preincubated for 1 hour in amino acid -^{14}C labeled mixture. After addition of HE or CE, incubation was continued for 90 min. Nuclear mitochondrial supernatants were prepared from the 2 groups, treated with DOC, and layered directly onto a sucrose gradient (see "Methods" for further details). OD_{260} (●); cpm (×).

we have established that hypothalamic extracts similar to those used in the present study are effective in promoting release of gonadotropin (21). This finding is also supported by others (7, 22).

When the experiments shown in Fig. 3 were repeated with amino acid-^{14}C mixture, no statistically significant difference between the extracts was found, although incorporation rates were consistently higher in HE-treated glands. It is likely that the effect of HE is on a relatively small fraction of protein synthesized in the pituitary system, and when labeled amino acid mixture is used, the stimulatory effect is obscured. Wool *et al.* (2) noted a similar effect in their studies dealing with pituitaries from adrenalectomized animals.

Preliminary experiments designed to test the effects of HE and CE on pituitary polyribosomes have been carried out. The OD profiles of DOC-treated mitochondrial supernatant from glands incubated with amino acids-^{14}C and HE or CE for 90 min are shown in Fig. 4. Differences in the absorbancy patterns are minor; however, the relative specific activities of the material in the polysome regions of the gradient are quite different. Thus, considering fractions "R"–11, the cpm/OD_{260} for HE and CE treatments were 4310 and 2430, respectively. Since relative specific activities of the finished proteins (fractions 12–15) were virtually identical, the results tend to strengthen the idea that HE stimulates *release* of some newly synthesized protein.

In conclusion, the results demonstrate the stimulatory effect of hypothalamic extracts on protein synthesis in pituitaries *in vitro*. Since HE does not alter the rate of RNA synthesis in this system (21), we tentatively conclude that HE acts at the cytoplasmic

level. We are currently investigating the effects of HE on synthesis of *specific* protein hormones in an organ culture system.

Summary. Rat anterior pituitary glands cultured for periods up to four hours incorporate amino acid-^{14}C mixture or glutamic acid-^{14}C into pituitary protein in linear fashion. Addition of hypothalamic extracts to the culture system results in an increased incorporation and release of protein radioactivity as compared to glands incubated with cerebral cortex extracts. The stimulatory effect can be demonstrated at the level of the pituitary polyribosomes.

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Isolation of Human Enteroviruses from Beagle Dogs* (33038)

D. L. LUNDGREN, W. E. CLAPPER, AND A. SANCHEZ

Department of Microbiology, Lovelace Foundation for Medical Education and Research, Albuquerque, New Mexico 87108

Dogs are one of the most common household pets and have more intimate contact with man than other domestic animals. It is, therefore, not unexpected to find that agents such as mumps virus (1), reovirus (2,3), and ECHO virus (4) which typically infect man have been isolated from dogs. Such observations indicate the probability that other viruses causing disease in man may be found, at least as transients, in dogs. The studies reported here were initiated to determine whether these or other viruses could be recovered from beagle dogs maintained in an isolated colony for other experimental purposes. Several isolations of ECHO virus were again made, as well as multiple isolations of Coxsackie B viruses.

Materials and Methods. Animals. The beagle dogs used were reared and maintained in kennels which housed a total of approximately 800 animals during the course of this study. Compatible pairs of dogs were housed in runs with concrete floors and were separated from other dogs by a cinder-block wall and wire fence. In some instances dogs were housed individually in metabolism cages.

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