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Effect of Hypothalamic Extracts on RNA Synthesis in Rat Anterior Pituitary Tissue.* (32299)

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Addition of crude acid extracts of rat hypothalamus to rat anterior pituitary glands in organ culture results in release of biologically detectable quantities of trophic hormones (*e.g.*, TSH, FSH) within 2 to 6 hours of incubation(1-5). This observation has provided experimental evidence for the concept of neuroendocrine regulation of anterior pituitary gland function. Thus, peptide "releaser" hormones, produced within specific neurosecretory cells of the hypothalamus, reach cells of the anterior pituitary gland by way of the hypophyseal portal circulatory system. These substances then cause the *release* of preformed hormone and, probably, the *synthesis* of new hormone(1,2).

It is generally believed that some hormones may, as a primary event, activate transcriptive mechanisms within the nucleus of the target cell(6-8). A study of RNA synthesis in explants of rat anterior pituitary tissue *in vitro* was undertaken in an effort to determine if, in addition to their primary function of release, hypothalamic extracts affect the rate of RNA synthesis in pituitary cells. The results presented here indicate that molecules of the ribosomal precursor and ribosomal type (as well as populations of heterogeneously labeled molecules) are actively synthesized in the pituitary organ culture system. They further show that the rate of incorporation of uridine-H^3 into RNA is not increased above control levels in glands exposed to hypothalamic extracts *in vivo* or *in vitro*.

Materials and methods. Preparation of pituitaries. Adult male rats (170-300 g), obtained from the Holtzman Co., Madison, Wis., were used in these studies. They were maintained on a 12-hour light, 12-hour dark cycle with feeding *ad lib*. Animals were sacrificed by cervical dislocation, pituitary glands removed and the posterior lobes discarded. Twelve to fourteen animals were usually required for each experiment. Incubation conditions were essentially the same as those used by Meites and his co-workers(1-4). Synthetic medium 199 (Difco Labs), buffered with $NaHCO_3$ to pH 7.2-7.4, was used. One or two anterior pituitary glands (halves or quarters) were incubated (Dubnoff shaker, 60 cycles/minute, 37°C) in 10 ml Erlenmeyer flasks containing 1.5 ml of medium/gland under a 95% O_2-5% CO_2 atmosphere.

Preparation of hypothalamic extracts. Fresh whole hypothalamic tissue from 12-14 rats was placed on ice, pooled, and homogenized in 1.2 ml of cold 0.1 N HCl with a ground glass homogenizer. Time from removal of fresh tissue to homogenization was 15 minutes. Homogenates were centrifuged in 0.6 ml

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capacity tubes at 105,000 *g* in the SW-39 rotor of the Model L Spinco ultracentrifuge for 30 minutes. Fresh acid extracts of an equivalent weight of pooled cerebral cortices were prepared in similar fashion.

Administration of extracts in vitro. The clear supernatants were neutralized with 1 N NaOH and added to the incubation medium containing the pituitary explants. Specific incubation conditions, *e.g.*, time, hypothalamic equivalents/pituitary, and time at which uridine-H^3 (New England Nuclear Corp.) and/or extract was added to the flasks, are given in the text. In all experiments uridine-H^3 (specific activity greater than 2 c/mM) was used at a concentration of 2 μc/ml of incubation medium.

Administration of extracts in vivo. In some experiments fresh acid extracts of hypothalamus (1.0 equivalent) were administered by intrajugular injection to male rats under ether anesthesia. Control animals received an equal volume (0.15 ml) of 0.1 N HCl by the same route. Animals recovered from anesthesia within 2-3 minutes after injection. The animals were sacrificed 20 to 45 minutes after injection, pituitaries removed and incubated as described above.

Nucleic acid extraction. Flasks containing pituitary explants were placed on ice immediately after incubation. After discarding the medium, the explants were washed twice in cold homogenizing medium (distilled water containing 2 mM $MgCl_2$) to remove excess uridine adsorbed to the surface of the explant. Homogenates were frozen and stored at −30°C for later analysis. Nucleic acids were extracted with perchloric acid as described by Kuff and Hymer(9). DNA content was determined using Giles and Myers' modification(10) of the Burton(11) procedure, with sperm DNA as the standard.

The sensitivity of the DNA test permits analysis of individual pituitary glands. Accordingly, rates of RNA synthesis are expressed on a DNA basis, *i.e.*, cpm in RNA/μg DNA.

RNA isolation. Total cellular RNA was extracted from pituitary glands according to the hot phenol-SDS method of Scherrer and Darnell(12). Ten pooled glands were used for each pulse period (Fig. 2).

In one experiment (Fig. 4), ribosomal RNA was extracted from pituitary ribosomes. A mitochondrial supernatant fraction (8700 *g*, 7 min) was obtained from a homogenate (0.25 M sucrose containing 2 mM $MgCl_2$) of 8 pituitaries incubated for 4 hours. This fraction was treated with sodium deoxycholate (0.5% final concentration) and centrifuged at 105,000 *g* for 2.5 hours. RNA in the ribosomal pellet was liberated from protein by treatment with 0.5% sodium dodecyl sulfate and the extract layered directly on a sucrose gradient as described below.

Sucrose gradient analysis and radioactivity assays. RNA extracts were layered on precooled 5-20% (w/v) linear sucrose gradients (volume 4.8 ml) containing either 0.05 M NaCl in 0.01 M sodium acetate, pH 5.1(12) (Fig. 3) or 1 mM $MgCl_2$ buffered with 0.01 M Tris Cl, pH 7.4(13) (Fig. 4). Centrifugation was done in the SW-39 rotor at a temperature of 4°C (see text for details). Three-tenths ml fractions were collected, diluted to 2.0 ml, read in a Beckman DU spectrophotometer and subsequently analyzed for radioactivity. $OD_{260/230}$ and $_{260/280}$ ratios were 2.0 or greater in the peak fractions.

Radioactivity was measured in a Nuclear Chicago liquid scintillation spectrometer (model 6850) using Bray's solution(14). Nucleic acid extracts were neutralized with NH_4OH prior to addition of the scintillation mixture. All samples were corrected to a uniform counting efficiency by the internal standard method.

Radioautography. Glands incubated for 4 hours in Medium 199 containing uridine-H^3 (2 μc/ml) were thoroughly washed in nonradioactive medium, fixed in formalin, embedded and sectioned at 5 μ. After application of NTB-3 Kodak liquid emulsion, slides were stored at 4°C in the dark for 21 days prior to development. Sections were stained in hematoxylin-eosin. No significant difference in labeling patterns was observed in those sections which were washed in 6% PCA prior to application of the emulsion. Labeled cells were observed throughout the glands.

Results. RNA synthesis in pituitary glands

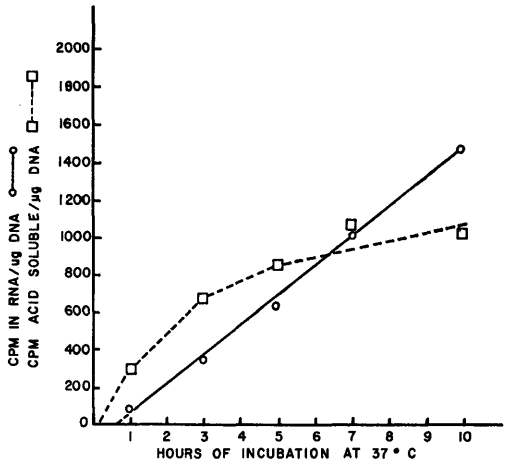

FIG. 1. RNA synthesis in anterior pituitary glands incubated *in vitro*. Results represent the average specific activities of 2 separate experiments in which each flask contained the equivalent of a single pituitary gland (in halves).

in vitro. The rate of incorporation of uridine-H_3 into the RNA of anterior pituitary gland halves incubated *in vitro* is shown in Fig. 1. After a short lag, synthesis was linear in glands incubated for 10 hours, the longest period thus far studied. The specific activities of the acid soluble fractions rose rapidly, followed by a gradual leveling-off phase (Fig. 1.). This observation indicates that synthesis is occurring at the expense of radioactive precursor in the intracellular pool. It is important to note that after 4 hours of incubation, the longest time period used in the remainder of the experiments presented here, less than 2% of the total acid precipitable counts in the pituitary homogenates were recovered in the DNA fraction obtained by the Schmidt-Thannhauser extraction procedure(15).

Histological and radioautographic evidence for cell viability. Histological examination of explants taken from 10-hour incubates revealed relatively few non-viable cells, in confirmation of an earlier report by Meites *et al*(16).

Radioautographs of sections prepared from glands incubated for 4 hours indicated that the cells at the periphery of the explants were heavily labeled (Fig. 2A). The cells in the central regions of the explants were less heavily labeled (Fig. 2B). Since there does not appear to be a preferential uptake of label into a specific cell type, it is suggested that the results obtained by the biochemical procedures reflect RNA synthesis in all pituitary cell types.

Molecular species of RNA synthesis in pituitaries in organ culture. Sucrose gradient analyses of total pituitary cellular RNA extracted from pooled glands after 30, 60, 120, 240 minutes of incubation are shown in Fig. 3. On the basis of earlier cosedimentation analyses with pituitary and liver RNA extracts, the nature of the OD_{260} material in fractions 8-9 and 10-11 can be identified as 28S and 18S ribosomal RNA. The material in the upper portion of the gradient is presumably, in part, transfer RNA.

Radioactivity patterns (Fig. 3) are typical of those obtained for other mammalian cell

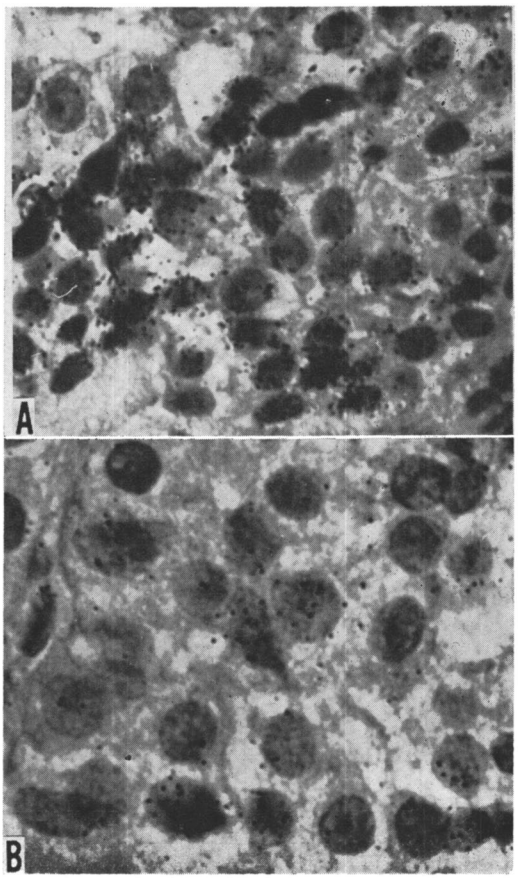

FIG. 2. Radioautographs of sections prepared from pituitary halves incubated for 4 hrs in Medium 199 containing uridine-H^3 (2 µc/ml).

FIG. 2A. Cells near periphery of explant, ×1133. 2B. Cells in central region of explant, ×1380.

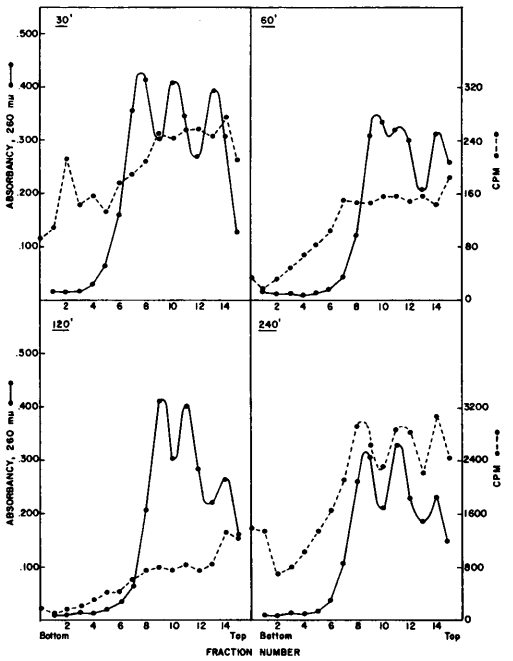

FIG. 3. Sucrose density gradient profiles of total cellular RNA prepared from pituitary glands incubated for 30, 60, 120 and 240 mins. in Medium 199 containing uridine-H^3 (2 μc/ml). Ten pituitary glands were used for each pulse period. Note the change in CPM scale in the 2 and 4 hr periods. Centrifugation conditions as follows: 30' pulse, 4 hrs at 37,000 R.P.M.; 60' pulse, 3.25 hrs at 35,000 R.P.M.; 120' pulse, 4 hrs at 35,000 R.P.M.; 240' pulse, 4 hrs at 36,000 R.P.M.

systems(13,17,18). Thus, in the early pulses, predominant labeling is found in material heavier than 28S ribosomal RNA. On the basis of other work(17,18), these molecules are presumably of the ribosomal-precursor type. A heterogeneous pattern of label was observed throughout the rest of the gradient (30- and 60-minute patterns), After the 2-hour pulse, however, labeling of the ribosomal RNA is suggestive, and after 4 hours readily apparent.

In one experiment, cytoplasmic ribosomes were isolated from 8 pituitary glands after 4 hours of incubation. The RNA pattern (Fig. 4) indicates that newly synthesized ribosomal RNA is present in cytoplasmic ribosomes. The relatively high specific activity of the material in the 18S peak is of interest, and has also been observed in other mammalian systems(9,19).

Effect of hypothalamic extracts on pituitary RNA synthesis in vitro. *Cerebral cortex extract controls.* The results of 3 separate experiments designed to compare the rate of RNA synthesis in pituitaries treated with neutralized hypothalamic extracts (H.E.) *in vitro vs.* corresponding controls treated with neutralized cerebral cortex extracts (C.E.) are shown in Table I. Although the rate has not been studied under conditions of long term incubation, it is clear that the initial rates in control (C.E.) and experimental (H.E.) glands are very similar.

Effect of hypothalamic extracts on pituitary RNA synthesis in vitro. *Administration of extracts* in vitro; *HCl controls.* The results of 7 experiments in which 0.1 N HCl served as the control treatment are presented in Table II. In 4 of these experiments (Table II-A), uridine-H^3 and neutralized HCl (control) or neutralized hypothalamic extract (experimental) were added to the flasks at zero time, followed by incubation periods of 1, 2, 4 hours. A decreased rate of precursor incorporation into RNA was consistently observed in the experimental group in the later incubation periods. After 4 hours this depression was significantly lower ($p<.05$) than controls.

In 3 experiments (Table II-B), all glands were preincubated for 2 hours in Medium 199 containing uridine-H^3. After addition of 0.1 N HCl or hypothalamic extract, incubations were continued for 1, 2 and 4 hours. In these cases, incorporation rates in the ex-

TABLE I. Effect of Hypothalamic (H.E.) *vs* Cerebral Cortex (C.E.) Extracts on RNA Synthesis in Rat Anterior Pituitary Glands Incubated *in vitro*.

Exp No.	Incubation time (hr)	Tissue equivalents/pituitary gland	epm in RNA/μg DNA H.E.	C.E.
1*†	1	.3	143	182
	4	.3	584	610
2	1	1.6	107	129
	4	1.6	343	328
3	1	1.6	98	92
	3	1.6	301	227
	5	1.6	402	502

* Each flask contained the equivalent of 2 rat anterior pituitaries (in quarters).

† Hypothalamic extract (H.E.) or cerebral cortex extract (C.E.), together with uridine-H^3, added at start of incubation period.

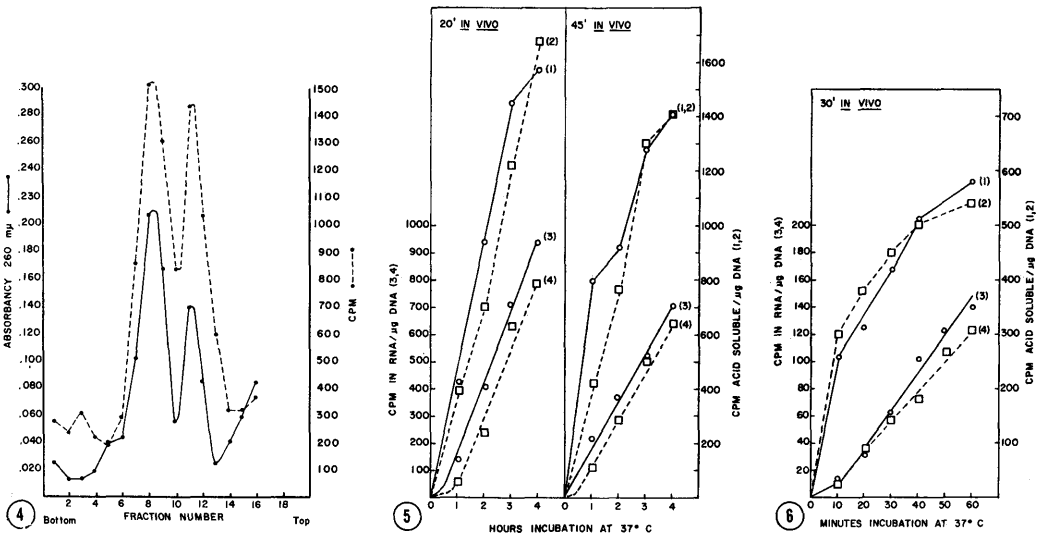

FIG. 4. Sucrose gradient sedimentation patterns of RNA extracted from a ribosome fraction isolated from 8 pituitary glands incubated *in vitro* for 4 hrs in Medium 199 containing uridine-H^3 at a concentration of 2 μc/ml. Centrifugation conditions: 4 hrs at 37,000 R.P.M.

FIG. 5. *In vitro* incorporation of uridine-H^3 into pituitary glands of 16 animals injected (intrajugular) with 1 hypothalamus equivalent (experimental) or 0.1 N HCl (control) 20 and 45 mins. prior to sacrifice. Acid soluble fractions: (1) control O———O, (2) experimental □———□, RNA fraction: (3) control O———O, (4) experimental □———□. For conditions of incubation, see text.

FIG. 6. *In vitro* incorporation of uridine-H^3 into pituitary glands of 12 animals injected with 1 hypothalamus equivalent (experimental) or 0.1 N HCl (control) 30 min prior to sacrifice. See legend, Fig. 5 for details.

perimental and control groups were identical for the first 2 hours of incubation, and a slight decrease was observed in the experimental group at the 4-hour period.

These results strongly suggest that the apparent decreased rate of RNA synthesis can be explained on the basis of a lower rate of incorporation of radioactive precursor into the intracellular pools of the H.E. treated glands. Although the experiments do not provide insight into the actual mechanism, it appears certain that the result cannot be explained on the basis of an *in vitro* "coating" phenomenon (see below).

Effect of hypothalamic extracts on pituitary RNA synthesis in vitro. *Administration of extracts* in vivo. Recent studies have shown that depletion of pituitary hormones *in vivo* occurs approximately 30 minutes after intrajugular or intracarotid injection of hypothalamic extracts(20,21). Experiments were conducted to determine the effect of injection of acid hypothalamic extracts *in vivo* on rates of incorporation of uridine-H^3 into pituitary gland RNA *in vitro*. The results of 2 separate experiments of this type are shown in Fig. 5. Pituitaries removed from animals sacrificed 20 and 45 minutes after injection of the equivalent of 1 hypothalamus/animal showed, for the first few hours of incubation, a lower rate of uridine-H^3 incorporation into the glands (*cf.* specific activities of acid soluble pools) compared with corresponding control animals injected with an equivalent volume of 0.1 N HCl. As reflected in the slopes of the lines, the rate of RNA synthesis was, however, the same in both groups (*cf.* specific activities of RNA, *i.e.,* cpm in RNA/μg DNA). It is suggested that the lag in the experimental groups (Fig. 5) can be explained on the basis of an initial decreased rate of uptake of radioactive precursor into the glands from the H.E. treated animals.

To establish more precisely the earliest time at which this lag could be observed, glands taken from animals injected with H.E. or HCl 30 minutes prior to sacrifice were incubated *in vitro* for 10-minute intervals up to 1 hour.

TABLE II. Effect of Hypothalamic Extracts on RNA Synthesis in Rat Anterior Pituitary Glands Incubated *in vitro*.

A. Uridine-H^3 and hypothalamic extract* (experimental) or neutralized HCl (control) added at start of incubation period.

Incubation time (hr)	No. animals	cpm in RNA/μg DNA
1 hr—control†	8	237.6 ± 47.2‡
1 " —experimental	8	261.1 ± 65.5
2 " —control	8	791.5 ± 142.1
2 " —experimental	8	506.4 ± 168.1
4 " —control	8	1408.3 ± 103.4
4 " —experimental	8	1077.7 ± 14.9§

B. Pituitary glands preincubated for 2 hr in Medium 199 containing uridine-H^3. After this period hypothalamic extract (experimental) or neutralized HCl (control) added and incubated as indicated.

Incubation time (hr)	No. animals	cpm in RNA/μg DNA
2 hr—preincubation	6	262.0 ± 25.6
1 " —control	6	356.3 ± 30.6
1 " —experimental	6	359.7 ± 100.6
2 " —control	6	501.2 ± 83.1
2 " —experimental	6	510.0 ± 108.8
4 " —control	6	965.3 ± 303.3
4 " —experimental	6	883.7 ± 375.7

* The equivalent of 1 hypothalamus/pituitary gland was used in all flasks designated "experimental."

† Each flask contained the equivalent of 2 rat anterior pituitary glands (in quarters) for each experiment. II-A, 4 experiments; II-B, 3 experiments.

‡ Standard error of the mean specific activities.

§ Indicates value significantly different from control ($p < .05$).

The rate of RNA synthesis was approximately the same for the first 30 minutes, but was less in the experimental group after this time (Fig. 6). In addition, the rate of incorporation of radioactive precursor into the glands was slightly higher for the first 30 minutes in the experimental group, but was lower than control values after this time.

The results of preliminary experiments indicate that the lag in uptake of uridine-H^3 is no longer seen in glands taken from animals injected with H.E. 3.5 hours prior to sacrifice; RNA synthetic rates in the two groups (H.E. *vs.* HCl) are very similar.

Discussion. The results presented here clearly demonstrate that RNA synthesis is occurring in rat anterior pituitary glands in organ culture. Radioautographs, in conjunction with sucrose density gradient centrifugation patterns of rapidly labeled RNA, indicate the synthesis of macromolecular RNA.

It should be pointed out that the hypothalamic extracts used in these studies were tested for their ability to effect release of pituitary gonadotropins *in vitro*. Although a relatively insensitive ovarian weight assay (22) was used, a decreased gonadotropin content in the gland, and a corresponding increase in activity in the incubation medium, was found in those glands treated with hypothalamic extracts. These observations are consistent with other reports (3,21) using more sensitive assay methods.

It should also be mentioned that the entire spectrum of RNA molecules was found to be synthesized in glands treated with hypothalamic extracts *in vitro*, albeit at a rate which was lower than corresponding HCl treated controls. The specific activities (cpm/OD_{260}) of all RNA fractions were uniformly reduced in the experimental group, thus ruling out the possibility of stimulation of synthesis of a *specific* set of molecules at the expense of others in the H.E. treated glands.

It is not possible to determine in these experiments whether the hypothalamic extracts, when injected into the animals, had a *direct* effect on the pituitary gland. However, the similarity of the results obtained in the *in vitro* and *in vivo* experiments (Table II, Fig. 5) imply that this may be the case. Taken together, these results do not support the hypothesis that hypothalamic releaser substances, as a primary event, activate specific transcriptive processes within pituitary cells.

An interesting report by Corbin and Story (20) suggests that maximum release of pituitary FSH occurs 45 minutes after intrajugular injection of hypothalamic extracts. The hormone is then resynthesized until content in the gland approaches control levels approximately 4 hours later. In this connection, it is also of interest that active protein synthesis in cell-free systems consisting of pituitary ribosomes from the bovine and murine species has recently been reported (23,24).

The results presented here seem consistent

with the idea that specific messenger RNA molecules in pituitary cells may be relatively long-lived. In an effort to further clarify those subcellular events which occur prior to hormone secretion, we are currently investigating the effects of hypothalamic extracts on protein synthesis in rat pituitary tissue.

Summary. 1. Evidence obtained by radioautography and sucrose density gradient centrifugation indicated that macromolecular RNA was synthesized in explants of rat anterior pituitary tissue *in vitro*. 2. The rapidly labeled RNA was, in part, heavier than 28S. 3. After 4 hours of incubation, label was observed in cytoplasmic ribosomal RNA. 4. The rate of RNA synthesis in glands treated with hypothalamic or cerebral cortex extracts *in vitro* was found to be similar. 5. The decreased rate of RNA synthesis in pituitaries treated with hypothalamic extracts *in vitro* (*vs.* corresponding controls treated with 0.1 N HCl) could be partially overcome by preincubating the glands in radioactive Medium 199 prior to addition of the extracts. 6. The *in vitro* rates of RNA synthesis in pituitaries taken from animals previously injected with hypothalamic extracts or 0.1 N HCl were similar, although an initial lag in uptake of uridine-H^3 into the glands of the experimental group was observed.

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Acoustic Profile of Deglutition. (32300)

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During a series of studies on the physiology of swallowing, it became necessary to obtain a precise index of the occurrence of deglutition. Electromyography was among the several forms of energy detection which were investigated. Potentials were recorded from the anterior surface of the neck with bipolar surface electrodes. Although discrete signals were obtained during deglutition, this method was not found suitable for general use. A promi-