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HYPOTHALAMO-NEUROHYPOPHYSIAL INVOLVEMENT IN THE CORTICOTROPHIC ACTION OF ACETYLCHOLINE

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Acetylcholine produced a fall of blood eosinophils and adrenal ascorbic acid in normal rats; these effects did not occur in hypophysectomized animals or in hypophysectomized animals bearing a pituitary homotransplant in the anterior chamber of the eye. Acetylcholine had a striking and long-lasting antidiuretic effect in normal rats, which was abolished by hypophysectomy. In normal dogs, intravenous injections of acetylcholine raised the concentration of posterior pituitary antidiuretic hormone in the cerebrospinal fluid. These results are discussed in connexion with the hypothesis that posterior pituitary antidiuretic hormone may be the neurohumoral substance involved in the hypothalamic control of the anterior pituitary.

Although the secretion of adrenocorticotrophic hormone (ACTH) may be controlled by the nervous system, there is little information available about the details of the mechanism involved.

There is no histological evidence for the direct hypothalamic innervation of the anterior lobe of the pituitary gland. For example, the most recent studies have failed to reveal any hypothalamic nerve fibres in the " pars distalis " (Harris, 1955). The view that the pituitary stalk links the hypothalamus to the adenohypophysis by means of the blood flowing down through the hypophysial portal vessels is supported by many observations. It has been suggested that a humoral substance, liberated by the hypothalamic nerve fibres into the primary plexus of the portal vessels in the median eminence, may reach the anterior lobe and excite the glandular cells (Harris, 1955).

Several suggestions have been put forward as to the nature of this chemical transmitting agent, such as adrenergic substances (Markee, Sawyer, and Hollinshead, 1948), histamine (Harris, Jacobsohn, and Kahlson, 1952), serotonin (Bertelli, Cantone, and Martini, 1954), substance P (Pernow, 1953), lipoid or lipoprotein present in hypothalamic extracts (Slusher and Roberts, 1954), and neurosecretory material produced in the hypothalamo-posthypophysial system (Benoit and Assenmacher, 1953; Palay, 1953; Rothballer, 1953).

In previous work, evidence was obtained supporting the view that the antidiuretic hormone (ADH) produced in hypothalamic nuclei may be considered as the neurohumoral substance involved in the hypothalamic control of the anterior pituitary (Martini and Morpurgo, 1955; Martini and De Poli, 1956; Martini, De Poli, and Curri, 1956).

The present investigation is concerned with the possible rôle played by acetylcholine (ACh) in the ACTH stimulating mechanism. Many observations seem to suggest that ACh may be an important factor in the pituitary activation by neurotropic stimuli. ACh occurs in high concentration in the hypothalamus (Macintosh, 1941; Feldberg, Harris, and Lin, 1951). The synthesis of ACh occurs in hypothalamic nuclei (Feldberg and Vogt, 1948). Moreover, many emotional stimuli associated with ACTH liberation may raise the ACh concentration in blood (Diethelm, Fleetwood, and Milhorat, 1950).

Experiments were planned to ascertain whether ACh could stimulate the release of ACTH from the anterior pituitary and to find whether the adrenocorticotrophic effect of ACh could be considered to be due to a direct action on hypophysial glandular cells rather than an indirect action involving the stimulation of the hypothalamic-neurohypophysial system.

METHODS

Animals and Drugs.—Male rats of the Sprague-Dawley strain, weighing 150 to 200 g., were used in the ACTH-releasing experiments and in the anti-

diuretic assays ; they were maintained on commercial diet and tap water. Dogs were used in the experiments studying the action of ACh on the concentration of ADH in the cerebrospinal fluid.

ACh was usually dissolved in 0.9% NaCl solution ; control animals were injected with corresponding volumes of 0.9% NaCl solution.

The significance of the differences between the means of the results obtained was calculated by Student's " t " test.

Hypophysectomy and Pituitary Transplantation.—Hypophysectomy was performed in rats by the standard parapharyngeal approach ; the hypophysectomized animals were used 5 days later.

In a group of animals the procedure of introducing adenohypophysial transplants into the anterior chamber of the right eye was carried out under light ether anaesthesia, animals of the same sex and weight serving as donors ; in this group, hypophysectomy was performed two days after grafting. Tests on the grafted rats were begun 50 days after hypophysectomy, ACh being given either by intraperitoneal injection or by local application to the graft-containing eyes by means of subconjunctival injections.

Eosinophils and Ascorbic Acid Depletion Test.—Tests were performed in normal rats, in hypophysectomized rats and in hypophysectomized rats bearing adenohypophysial homotransplants in the anterior chamber of the eye.

Immediately before, 2, and 4 hr. after giving intraperitoneal injections of ACh, direct counts of the eosinophils in the blood were made by the method of Recant, Forsham and Thorn (1948) on blood samples taken from the tail. Although it is recognized that this test is not a specific index of increased adrenocortical function, it is certainly very sensitive and was therefore adopted as a guide.

The results were subsequently confirmed with the ascorbic acid depletion test (Sayers, Sayers and Woodbury, 1948). The rats were killed by decapitation 3 hr. after treatment, the adrenals removed, freed from excess adipose and connective tissue, weighed on a torsion balance, and their content of ascorbic acid was estimated by the method of Roe and Kuether (1943).

Antidiuretic Test.—The antidiuretic action of ACh was studied both in normal and in hypophysectomized rats, after hydration according to Ginsburg's (1951) procedure. The percentage water excretion 60, 90 and 120 min. after the intraperitoneal injection of ACh was calculated ; this is inversely related to the antidiuretic power of the drug injected.

Antidiuretic Hormone Level in Dog Cerebrospinal Fluid.—The antidiuretic power of dog cerebrospinal fluid was determined on samples obtained from unanaesthetized animals by suboccipital puncture. The first sample (1 ml.) was obtained just before the intravenous injection of ACh in the test animals, and of 0.9% NaCl solution in the control animals. The second sample (1 ml.) was obtained 5 min. later through the same needle which had remained inserted. The antidiuretic power of these samples was tested by injecting subcutaneously 0.1 ml. of the cerebrospinal fluid into hydrated rats according to Ginsburg's (1951) procedure. Inactivation with sodium thioglycollate (Van Dyke, Chow, Greep and Rothen, 1942) was carried out in order to make sure that the antidiuretic activity of cerebrospinal fluid samples was due to posterior pituitary ADH.

Results

Effect of Acetylcholine on the Number of Circulating Eosinophils and on Adrenal Ascorbic Acid in Normal, Hypophysectomized, and Hypophysectomized Rats Bearing a Pituitary Graft in the Anterior Chamber of the Eye.—In normal rats, the intraperitoneal injection of ACh (1 mg./rat) was followed by a highly significant fall ($P<0.001$) in the number of the circulating eosinophils ; no corresponding change in the eosinophil blood count occurred in hypophysectomized or in grafted animals (Fig. 1).

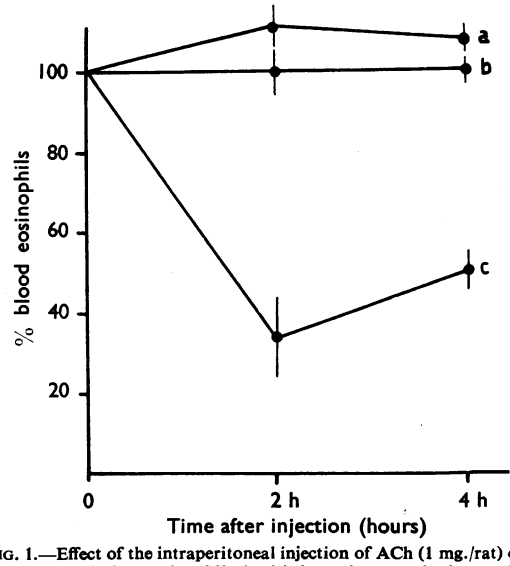

FIG. 1.—Effect of the intraperitoneal injection of ACh (1 mg./rat) on the circulating eosinophils in (a) hypophysectomized rats, (b) hypophysectomized rats bearing intraocular pituitary grafts, and (c) normal rats. Each point represents the mean of 10 experiments; the vertical lines indicate the standard error.

Similar results were obtained with the ascorbic acid depletion test. In normal rats, the intraperitoneal injection of ACh (1 mg./rat) resulted in a significant fall ($P<0.001$) in adrenal ascorbic acid. In hypophysectomized rats the same dose of ACh did not cause any depletion of ascorbic acid. Both by the intraperitoneal route (1 mg./rat), and by subconjunctival injection into the graft-containing eyes (30 μg./rat), ACh was ineffective in the grafted animals (Table I).

TABLE I

EFFECT OF INTRAPERITONEAL AND SUBCONJUNCTIVAL INJECTIONS OF ACETYLCHOLINE ON ADRENAL ASCORBIC ACID IN NORMAL RATS, HYPOPHYSECTOMIZED RATS AND HYPOPHYSECTOMIZED RATS BEARING INTRAOCULAR PITUITARY GRAFTS

No. of Animals	Treatment	Adrenal Ascorbic Acid (mg./100 g. Tissue ± Standard Error)		
		Normal	Hypophysectomized	Hypophysectomized and Grafted
10	NaCl 0·9% ..	432·1±25·1	422·5±18·5	452·3±15·0
10	Acetylcholine (1 mg./rat intraperitoneally)	325·0±13·5	445·0±22·4	427·5± 8·0
10	Acetylcholine (30 μg./rat subconjunctivally)	—	—	458·0±11·5

These experiments indicated that ACh exerted a marked corticotrophic effect, which was mediated through the activation of the anterior pituitary gland. The results obtained in the hypophysectomized animals bearing pituitary transplants showed that adenohypophysial cells did not respond in a direct way to ACh administration. Since the responsiveness of the pituitary gland to the drug depended upon the integrity of normal hypothalamo-hypophysial pathways, the action of ACh must take place at a higher level, probably in the hypothalamus. The stimulation of the hypothalamic-neurohypophysial system by ACh was then studied.

Effect of Acetylcholine on Water Diuresis in Normal and Hypophysectomized Rats.—ACh, when injected intraperitoneally (1 mg./rat) into hydrated normal rats, has a striking and long-lasting antidiuretic effect (Fig. 2). The drug has no antidiuretic effect in hypophysectomized rats in which, however, the elimination of the water-load is slower than in normal animals. As these two results appeared very similar in their characteristics to those obtained after giving posterior pituitary gland preparations, it seemed that the antidiuretic effect observed after ACh administration might have been due to a discharge of antidiuretic hormone from the hypothalamic-neurohypophysial system. This hypothesis was tested in the following experiments in which the concentration of ADH in the cerebrospinal fluid was measured in normal dogs before and 5 min. after giving intravenous injections of ACh.

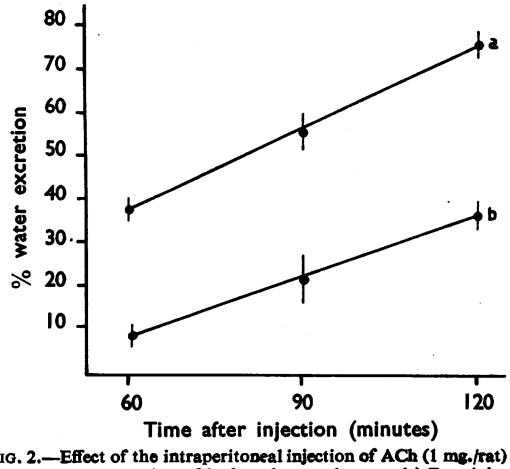

FIG. 2.—Effect of the intraperitoneal injection of ACh (1 mg./rat) on the water excretion of hydrated normal rats. (a) Rats injected with 0.9% NaCl solution. (b) Rats injected with ACh. Each point represents the mean of 10 experiments; the vertical lines indicate the standard error.

Effect of Acetylcholine on the Cerebrospinal Fluid Levels of ADH in Normal Dogs.—Fig. 3 summarizes the results obtained on injecting subcutaneously three groups of hydrated rats (*a*) with 0.1 ml. of 0.9% NaCl solution, (*b*) with 0.1 ml. of cerebrospinal fluid taken from three normal dogs before ACh administration, and (*c*) with 0.1 ml. of cerebrospinal fluid taken from the same three dogs 5 min. after the intravenous injection of 2 mg./kg. of ACh. The antidiuretic power of the cerebrospinal fluid was significantly enhanced after ACh administration.

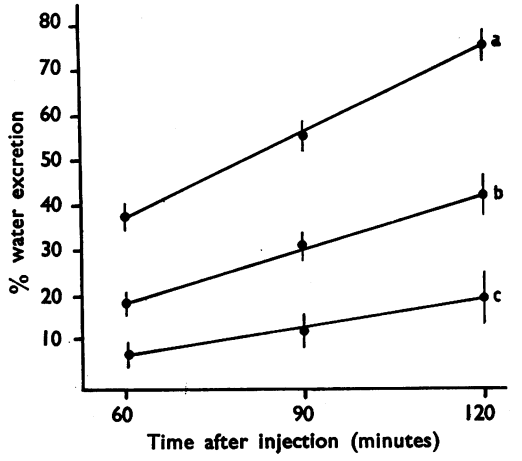

FIG. 3.—The water excretion in hydrated normal rats after the subcutaneous injection of cerebrospinal fluid (0.1 ml./rat) taken from normal dogs before and 5 min. after the intravenous injection of 2 mg./kg. of ACh. (a) Rats injected with 0.9% NaCl solution. (b) Rats injected with cerebrospinal fluid taken before ACh administration. (c) Rats injected with cerebrospinal fluid taken after ACh administration. Each point represents the mean of 15 experiments performed with cerebrospinal fluid taken from 3 dogs; the vertical lines indicate the standard error.

In our experiments the concentration of ADH in the cerebrospinal fluid of normal dogs (about

7.5 mU./ml.) was much higher than that found by Vogt (1953), namely <2.5 mU./ml.

After incubation with sodium thioglycollate, the antidiuretic activity of cerebrospinal fluid, withdrawn before or after giving ACh, was abolished in the same way as that of the posterior pituitary hormone after similar treatment.

In two dogs treated with 0.9% NaCl solution instead of with ACh, no enhancement of the cerebrospinal fluid level of ADH was observed.

Discussion

The results reported here indicate that ACh can stimulate the anterior pituitary to discharge ACTH. This is in agreement with the observation of Hofmann-Credner (1953) that ACh injections produced eosinopenia in human beings. Similarly, Guillemin (1955) found that methacholine lowered the adrenal ascorbic acid in normal rats. Working on the same lines, Dordoni and Fortier (1950) and Dordoni and Timiras (1952) have shown that in intact animals a single injection of eserine depleted the adrenal ascorbic acid and that prolonged treatment with eserine produced marked hypertrophy of the adrenals and atrophy of the thymus.

These studies of the ability of parasympathomimetic drugs to release ACTH have an interesting parallel in those of Taubenhaus and Soskin (1941), which showed that injection of ACh resulted in the production of pseudopregnancy in the rat, that is, in the release of luteotrophic substance (lactogenic hormone) from the anterior pituitary. According to Markee, Everett, and Sawyer (1952) ACh also plays an important rôle in the liberation of enough luteinizing hormone (L.H.) to cause ovulation.

As to the site and mode of action of ACh as an ACTH-releaser, the present experiments have clearly shown that it has no direct stimulating action on adenohypophysial glandular cells; ACh is thus unlikely to be the substance released by hypothalamic neurones into the hypophysial portal vessels under the influence of stress, and it is not responsible for an increase in the pituitary secretion of ACTH above the resting state. In agreement with these results, Feldberg and Vogt (1948) have shown that there are only small amounts of the ACh-forming enzymes in the pituitary gland; and they concluded that no cholinergic fibres originate from the cells in the supra-optic region.

As the integrity of normal hypothalamo-hypophysial connexions was needed for the action of ACh, it seemed that the drug might act on these pathways at the hypothalamic level. We have shown that, in rats and dogs after the administration of ACh, there is a stimulation of hypothalamic centres of neurones, which leads to the release of antidiuretic hormone from the hypothalamo-neurohypophysial system.

Using adrenaline, which also can release ACTH, Martini and Rovati (1952a, 1952b, 1956) obtained similar results.

Evidence that ACh may act on hypothalamic nuclei or neurones has been obtained also by Pickford and Watt (1951) and by Abrahams and Pickford (1954). These authors have shown that intravenous and intracarotid injections of ACh produced a marked antidiuretic effect in normal hydrated dogs, but failed to inhibit water diuresis after the production of diabetes insipidus by suitably placed hypothalamic lesions. ACh, injected directly into the supra-optic nuclei of normal dogs during a water diuresis, resulted in a marked inhibition of the rate of urine flow (Pickford, 1947); such an inhibition may be prolonged by adding eserine to the solution injected (Pickford, 1947). Eserine and DFP, when injected alone, produce a similar long-lasting antidiuretic effect (Duke, Pickford, and Watt, 1950); eserine and DFP when injected into the supra-optic nuclei of dogs anaesthetized with chloralose led to a release of oxytocin and to an increase in size of spontaneous uterine contractions (Abrahams and Pickford, 1956).

These experiments support the hypothesis that the action of ACh on the anterior pituitary is mediated through the release of antidiuretic hormone, or some related compound liberated at the same time as antidiuretic hormone, into the hypophysial portal vessels.

The early experiments of Martini and Morpurgo (1955), of Martini and De Poli (1956), and of Martini, De Poli, and Curri (1956). as well as more recent investigations, indicated that posterior pituitary preparations do, indeed, stimulate the release of ACTH. Sayers (1956) has recently shown that a commercial antidiuretic hormone preparation (Pitressin, Parke, Davis) and a more purified vasopressin sample (Du Vigneaud's AVN-5) exhibited a marked action causing the discharge of ACTH. McDonald and Weise (1956) and Shibusawa, Saito, Fukada, Kawai, and Yoshimura (1955) have shown that, in man, plasma 17-hydroxycorticosteroid levels were elevated during infusions of both commercial pitressin and purified antidiuretic hormone preparations (Du Vigneaud's arginine- and lysine-vasopressin). Working on the same lines, McCann and Brobeck (1954) have reported that lesions of the supra-optico-hypo-

physial tract, which produced diabetes insipidus, blocked the release of ACTH in response to stressor agents. In rats which, as a result of lesions in the median eminence, no longer responded to other stressors, ACTH secretion can be elicited by the administration of pitressin.

The physiological significance of the power of ACh to release ACTH is not yet clear; it should be remembered in this connexion that Guillemin (1955) has shown that in rats treated with atropine the release of ACTH which normally follows the injection of parasympathomimetic drugs was completely prevented, while ACTH was still liberated by systemic stress and neurotropic stimulation.

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