ACETYLCHOLINE IN BOVINE ANTERIOR PITUITARY, POSTERIOR PITUITARY, AND PINEAL

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ABBREVIATIONS AND SYMBOLS

Å	Ångstrom unit (10^{-8} centimeter)
ACh	acetylcholine
[ACh]	acetylcholine concentration
AChE	acetylcholinesterase
AP	anterior pituitary
AP-CZ	central zone of anterior pituitary
ASCh	acetylthiocholine
[atropine]	atropine concentration
BuCh	butyrylcholine
BuChE	butyrylcholinesterase
C	centigrade
ChAc	choline acetylase
ChE	cholinesterase
cm	centimeter
F	ratio of variances
g	gram
g	gravity
HY	hypothalamus
μC_i	microcurie
μl	microliter
M	molar (gram-molecular weight/liter)
mg	milligram
min	minute
ml	milliliter
mm	millimeter

mole	gram-molecular weight
N	normal (gram-equivalent weight/liter)
nmole	nanomole (10^{-9} gram-molecular weight)
NS	not significant
PB	pineal body
pH	negative logarithm (to the base 10) of the hydrogen ion concentration
PP	posterior pituitary
PrCh	propionylcholine
RB	rat brain
S. E.	standard error of the mean
Std.	standard
v/v	volume/volume
w/v	weight/volume

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ABSTRACT

Acetylcholine (ACh) tissue concentrations have been found for bovine anterior pituitary, posterior pituitary, and pineal body. Various extraction methods (formic acid-acetone; acetic acid-ethanol; perchloric acid) were compared. Tissue extracts were purified by paper electrophoresis or cation-exchange chromatography. The ACh concentration of purified tissue extracts was estimated by use of the isolated guinea-pig ileum preparation.

Anion-exchange chromatography (0.6 cm X 10.0 cm columns of Dowex 1-X8; chloride form) was used with the tissue extracts prior to isolation of ACh by either high-voltage paper electrophoresis or cation-exchange chromatography (0.6 cm X 12.0 cm columns of Bio-Rex 70; equilibrated with 0.1 M NaH_2PO_4). The eluting solvent for cation-exchange chromatography was 0.1 M NaH_2PO_4. The eluting solvents used for anion-exchange chromatography were 70/30 (v/v) methanol-water (prior to electrophoresis) or 50/50 (v/v) acetone-water (prior to cation-exchange chromatography). The anion-exchange chromatography removed anions that would interfere with the isolation and estimation of ACh.

The biologically active material of purified tissue extracts exhibited chemical characteristics (electrophoretic mobility and ion-exchange column

retention volume) identical to those of authentic ACh alone or added to the extracts. The active material, like ACh, was rapidly inactivated by boiling at pH 10 (but not at pH 4) or by incubation with purified bovine erythrocyte acetylcholinesterase (AChE). The guinea-pig ileum stimulating activity of purified extracts was completely abolished by a low concentration of atropine (2.9×10^{-8} M) known to abolish the guinea-pig ileum stimulating effect of ACh but not that of histamine. ACh alone or added to tissue extracts caused equal stimulation of the isolated guinea-pig ileum.

Bovine posterior pituitary extracts (acetic acid-ethanol) purified by paper electrophoresis were estimated to contain $2.3 \pm 0.2 \times 10^{-9}$ mole ACh/g of fresh tissue.

Formic acid-acetone or perchloric acid extracts of bovine posterior pituitary (cation-exchange chromatography purification) were estimated to contain $1.2 \pm 0.01 \times 10^{-9}$ mole ACh/g or $1.4 \pm 0.04 \times 10^{-9}$ mole ACh/g of fresh tissue, respectively. The lower estimates found for extracts purified by cation-exchange chromatography are attributed to the presence of sodium dihydrogen phosphate in the methanol extracts of lyophilized chromatography fractions. This salt depresses the isolated guinea-pig ileum response to ACh.

Whole bovine anterior pituitary (acetic acid-ethanol extract; paper electrophoresis purification) was estimated to contain $0.71 \pm 0.01 \times 10^{-9}$ mole ACh/g of fresh tissue. The central zone of the bovine anterior pituitary (formic acid-acetone extract; cation-exchange chromatography purification) contained $0.16 \pm 0.01 \times 10^{-9}$ mole ACh/g of fresh tissue.

Analysis of variance showed that differences among the mean ACh tissue concentrations were not significant for posterior pituitary ($F(2.12)$ 1.72) or for whole anterior pituitary ($F(1.7)$ 3.90). Thus sampling differences from one experiment to another were not indicated.

Extracts of bovine pineal body were purified by cation-exchange chromatography. Estimates of the ACh content of bovine pineal were $0.85 \pm 0.13 \times 10^{-9}$ mole/g (formic acid-acetone extract), $0.46 \pm 0.09 \times 10^{-9}$ mole/g (acetic acid-ethanol extract), and $0.36 \pm 0.14 \times 10^{-9}$ mole ACh/g of fresh tissue (perchloric acid extract). The highest value, found with formic acid-acetone extracts, was related to the sharper cation-exchange column resolution of ACh in formic acid-acetone extracts as compared with other types of extract. The cation-exchange column chromatography characteristics (retention volume and resolution) of perchloric acid extracts were inferior as compared with those of organic solvent extracts (formic acid-

acetone or acetic acid-ethanol), probably because of the presence of higher concentrations of extraneous salts in the perchloric acid extracts.

The ACh content of the cerebral hemispheres of hooded rats was estimated for acetic acid-ethanol extract (paper electrophoresis purification) and perchloric acid extract (cation-exchange chromatography purification) to be $6.2 \pm 0.8 \times 10^{-9}$ mole/g and $5.6 \pm 0.1 \times 10^{-9}$ mole ACh/g fresh tissue, respectively. An experiment with bovine hypothalamus indicated a tissue concentration of $1.4 \pm 0.1 \times 10^{-9}$ mole ACh/g of fresh tissue for the ACh-like material isolated from acetic acid-ethanol extract by paper electrophoresis.

The ACh content of bovine posterior pituitary is similar to the ACh content of bovine brain. Bovine posterior pituitary, pineal body, and anterior pituitary show a general parallelism of ACh content with the levels of activity of choline acetylase (ChAc) and AChE in the same tissues. The ACh content of these tissues also appears to show some parallelism with the extent of autonomic nerve supply that they receive. The physiological significance, as well as the tissue levels, of ACh, ChAc, and AChE may show marked species differences for the anterior pituitary, posterior pituitary, and pineal body.

I. INTRODUCTION

STATEMENT OF THE PROBLEM

This study gives a basis for comparison of the acetylcholine (ACh) content of three bovine tissues: posterior pituitary, anterior pituitary, and pineal body. The problem to be considered is two-fold: (a) Does ACh occur in appreciable concentrations in each of these tissues? and (b) What is the possible physiological significance of the ACh concentrations found for these tissues? The significance of the ACh concentrations must be evaluated in relation to the occurrence and levels of activity in these tissues of enzymes associated with ACh metabolism: choline acetylase (ChAc) and acetylcholinesterase (AChE).

The ACh concentrations of these tissues must also be considered in relation to the nature and extent of the nerve supply. The basic differences among these tissues are related to the different physiological significance of the nerve supply of each. The control of the function of these three tissues appears to be effected by three fundamentally different means, although endocrine function has been established or posulated for each of them.

The release of peptide hormones from the neurosecretory nerve terminals of the posterior pituitary is related to electrophysiological and chemical mechanisms analogous to those that control the release

of neurohumoral mediator substances from non-neurosecretory nerve fibers. The release of anterior pituitary hormones is controlled not directly by nervous impulses, but is controlled by blood-borne chemical mediators from nearby central nervous tissue. It is thought that the release of possible hormonal substances from the mammalian pineal body parenchymal cells may be under a type of secretomotor control exerted by the autonomic nerve fibers to the pineal.

Parasympathetic nerve fibers to the pineal body and anterior pituitary have been described in some mammalian species, and such parasympathetic fibers would presumably be cholinergic. However, no detailed hypothesis of a cholinergic mechanism of control of these tissues has been given serious consideration recently. For the mammalian posterior pituitary, an hypothesis of cholinergic control of peptide hormone release has been elaborated in recent years. The experiments that have tested this hypothesis are considered in the present study as they bear on the possible physiological significance of ACh in the posterior pituitary.

Marked species differences in type of innervation (presence or absence of catecholamine-containing fibers; relative importance of sympathetic and parasympathetic autonomic innervation) have been

described for the mammalian posterior pituitary, anterior pituitary, and pineal body. Marked differences have also been described in the AChE content of these tissues for various vertebrate species. Such differences must be given detailed consideration in connection with the possible physiological significance of ACh in the posterior pituitary, anterior pituitary, and pineal body.

NEUROSECRETORY NERVE FIBERS OF THE POSTERIOR PITUITARY

The neurosecretory neurons of the hypothalamo-hypophysial tract have their cell bodies in nuclei of the hypothalamus. Axons of this tract course through the infundibular stalk to reach the posterior lobe of the pituitary, where their terminals end, usually in close relation to the small blood vessels. The neuronal and the secretory characteristics of the posterior pituitary neurosecretory fibers are presented here as a basis for critical appraisal of the hypothesis that postulates a cholinergic mechanism to mediate posterior pituitary hormone release.

The concept of synthesis and release of peptide hormones (vasopressin and oxytocin) by neurosecretory neurons of the hypothalamo-hypophysial tract has replaced the concept that these neurons serve as secretomotor innervation for the pituicytes. The pituicytes are cells intrinsic to the neural lobe. They are variable in size and shape. The structural relationship of the pituicytes to the neurosecretory nerve fibers of the neural lobe is similar to that of neuroglia to neurons within the central nervous system (Bloom and Fawcett, 1968). Although the concept of peptide hormone synthesis and secretion by the pituicytes has been abandoned, the precise physiological role of the pituicytes remains to be

clarified (Sloper, 1966; Christ, 1966).

Secretory characteristics of the neurosecretory cells

The development of the concept of neurosecretion, prior to the use of electron microscopy, was based largely on the demonstration of histologically stainable features of certain neurons suggestive of glandular or secretory activity. Prior to 1949 no hypothesis had been experimentally established to relate explicitly the histological and physiological observations on the mammalian neurohypophysis to observations on the neurosecretory systems in lower animals. In 1949 W. Bargmann developed a stain that apparently demonstrated histologically the "neurosecretory material" of the neurohypophysis. It was soon established that such "neurosecretory material" characterized the hypothalamo-hypophysial tract of the entire range of vertebrates. In higher vertebrates this histologically demonstrable "neurosecretory material" was shown to be closely associated with the presence of the peptide hormones along the hypothalamo-hypophysial tract (Bargmann and Scharrer, 1951; Scharrer and Scharrer, 1954; Sloper, 1966; Green, 1966b).

The application of electron microscopic methods led to great refinement of the cytological criteria that could be employed in characterizing the neurosecretory neuron. Bargmann, Palay, and many

others (Palay, 1957; Bargmann, 1958; Green, 1966b; Bargmann and Gaudecker, 1969) have described electron-dense vesicles (so-called "secretory granules" or "elementary granules") in the neurohypophysis of vertebrates. Similar granules were found in the apparently neurosecretory neurons of many vertebrate and invertebrate species. Such findings gave rise to the expectation that the occurrence of such granules in axon terminals would conclusively indicate the occurrence of neurosecretion. It soon became apparent, however, that granules with such morphological appearance were present in many neurons where neurosecretion apparently was not (Bern and Knowles, 1966).

It should be pointed out that histological methods can reveal only the <u>possibility</u> of neurosecretory activity (Bern and Knowles, 1966). Experiments employing combinations of the techniques of electron microscopy, subcellular fractionation, biochemistry, and pharmacology have been necessary to establish the relationships between the histologically stainable "neurosecretory material" and the actual storage and release of the peptide hormones of the neurohypophysis.

<u>Association of the peptide hormones with "secretory granules"</u>

Pardoe and Weatherall (1955) studied the

subcellular distribution of vasopressin and oxytocin in homogenates of rat pituitary glands. They found some similarities between the sedimentation characteristics of mitochondria and those of the vasopressin and oxytocin. They inferred from this that vasopressin and oxytocin might be particle-bound. They obtained more complete sedimentation of vasopressin in 0.88 M sucrose, while oxytocin sedimented more completely in 0.25 M sucrose. They suggested that oxytocin and vasopressin might be contained in different particles in the posterior pituitary.

Subsequent investigations of the posterior pituitary employing electron microscopic, biochemical, and pharmacological methods have shown that vasopressin and oxytocin are not associated with mitochondria, but rather with granulated vesicles called "secretory granules." Results of the more recent work have, however, provided support for the suggestion of Pardoe and Weatherall (1955) that vasopressin and oxytocin may be stored in separate particles.

Heller and Lederis (1961) and Heller and Lederis (1962) studied the subcellular distribution of the peptide hormone activity of rabbit neurohypophysis. Weinstein, Malamed, and Sachs (1961) performed a similar study on the localization of the hormone activity of the neurohypophysis of the dog.

Electron microscopic observation of the fractions enriched in peptide hormone activity revealed the presence of the "secretory granules" that had previously been observed in nerve terminals of the intact neural lobe.

Evidence that vasopressin and oxytocin are contained in different particles with different sedimentation characteristics was obtained by Heller and Lederis (1961), Heller and Lederis (1962), LaBella, Beaulieu, and Reiffenstein (1962), LaBella, Reiffenstein, and Beaulieu (1963), and Barer, Heller, and Lederis (1963). LaBella and his co-workers found that vasopressin is associated with particles of greater density than that of oxytocin-containing particles. Lederis and his co-workers initially reported observations suggesting that oxytocin-containing particles might have lower density than vasopressin-containing particles. More recently, however, Lederis and Livingston (1968) have presented data that indicate the higher density for vasopressin-containing particles. Dean and Hope (1968) have confirmed the finding by LaBella and his co-workers that vasopressin-containing particles have the higher density.

LaBella and Sanwal (1965) were able to obtain bovine posterior pituitary subcellular fractions containing isolated, pinched-off nerve-ending particles.

These posterior pituitary nerve-ending particles are similar to the isolated nerve-ending particles that can be obtained from brain tissue by careful homogenization and centrifugation (Whittaker, 1965; De Robertis, 1967). Bindler, LaBella, and Sanwal (1967) characterized the nerve-ending particle fractions and other subcellular fractions from the bovine posterior pituitary in terms of ultrastructure, vasopressin and oxytocin concentrations, and enzyme activity. Bindler et al. (1967) obtained evidence that vasopressin-containing nerve-ending particles tended to show higher density than oxytocin-containing nerve-ending particles.

The findings from several different laboratories suggest that vasopressin and oxytocin may be contained in separate secretory granules, and perhaps in different nerve endings in the posterior pituitary. This appears to be a plausible suggestion since numerous physiological and pharmacological investigations have indicated preferential release of one hormone or the other with an appropriate stimulus (Heller, 1966; Heller and Ginsburg, 1966; Dyball, 1968).

Relationship of the peptide hormones to "neurosecretory material"

There is evidence to indicate that the concentrations of vasopressin and oxytocin may not be,

under all circumstances, closely associated with the amount of histologically demonstrable "neurosecretory material" in the posterior pituitary. Moses et al. (1963) found no change in vasopressin content to be correlated with decreased amounts of "neurosecretory material" in the rat neurohypophysis 90 minutes after formalin administration.

Lederis (1964) studied the ultrastructure and hormone content of the trout neurohypophysis. He found that exposing fresh-water trout to sea water for 2 hours led to a 50% decrease in the arginine-vasotocin content of the pituitary, although only a few nerve terminals contained vesicles devoid of electron-dense material. He suggested that lack of "neurosecretory material" or electron-dense granules is not necessarily related to absence of peptide hormone in the neural lobe.

Daniel and Lederis (1966b) investigated the effects of ether anesthesia and hemorrhage on the rat neurohypophysis, with simultaneous observations on hormone levels and ultrastructure of the same neural lobes. They found that depletion of hormones and increase in the proportion of "free" (i.e., not particle-bound) hormones were not sufficiently great to account for the almost complete depletion of electron-dense granules from most of the nerve fibers. They concluded

that it is not likely that there is a strictly quantitative relationship between stainable "neurosecretory material" or electron-dense material and the hormone content of the neural lobe.

It appears that, in the long term, there is some degree of positive correlation of neural lobe peptide hormone content with the amount of "neurosecretory material" or with the number of electron-dense "secretory granules" (Palay, 1957; Green, 1966b). In short-term experiments, however, the amount of "neurosecretory material" and the electron-density of granules is not to be equated with hormone content. This is an important point in regard to the development and testing of hypotheses about a possible cholinergic mechanism mediating posterior pituitary hormone release. The relative numbers of electron-dense "secretory granules" and smaller, electron-lucent, so-called "synaptic vesicles" have probably been relied on too heavily as an indication of the relative amounts of peptide hormone and ACh. The assumption has been that electron-density may be correlated with hormone-containing granules, while the presence of smaller, clear "synaptic vesicle"-like structures may be equated with the presence of ACh. Such assumptions, based on morphological observations without accompanying biochemical studies, are very tenuous.

Origin and significance of the "synaptic vesicle"-like structures

Palay (1957) found that prolonged substitution of 2.5% saline for drinking water leads to depletion of both the histologically demonstrable "neurosecretory material" and the electron-dense granules of the neurohypophysis. The nerve terminals of the neural lobe are filled with small, clear vesicles under such experimental conditions. Palay (1957) suggested that the small, clear vesicles in the nerve endings of the neural lobe may result from the breakdown of the larger, granulated vesicles ("secretory granules" or "elementary granules"). This interpretation might be applied in the case of neural lobe nerve terminals of unstressed individuals. Many of these terminals contain both the larger, electron-dense granules and the small, clear vesicles. It might also be applied in the case of nerve terminals in the neural lobe after prolonged stress has led to a marked increase in the number of small, clear vesicles and a decrease in the number of larger, electron-dense granules.

Some nerve terminals of the neural lobe in the normal, unstressed individual contain only the small, clear vesicles, however. Authors who have reported such observations (Daniel and Lederis, 1966b; Bindler et al., 1967; Lederis and Livingston, 1967)

have suggested that there may occur in the normal neural lobe some non-neurosecretory nerve terminals that do not contain secretory granules or peptide hormones. They have suggested, furthermore, that the small, clear "synaptic vesicle"-like structures in such non-neurosecretory nerve terminals might be associated with the storage of a neurohumoral agent such as ACh.

Thus there are two alternative interpretations that have been made regarding the origin and significance of the "synaptic vesicle"-like structures in the neural lobe. Such inclusions might be formed from the remnants of membranes of larger, electron-dense secretory granules, or they might be normally present as true "synaptic vesicles" containing a neurohumor such as ACh. Daniel and Lederis (1966b) have pointed out that there is not necessarily a contradiction between these two interpretations. Some of the small, clear vesicles in neural lobe nerve terminals might arise as "breakdown products" from the secretory granules, while others might be true "synaptic vesicles."

In view of the existence of an autonomic nerve supply to the neural lobe (Dandy, 1913; Christ, 1966), it is conceivable that at least some of the neural lobe nerve terminals containing only "synaptic vesicle"-like inclusions may be the terminals of cho-

linergic autonomic nerve fibers. There is, however, no evidence to rule out the possibility that, in some neurosecretory nerve terminals, the "synaptic vesicle"-like inclusions may arise as breakdown products of the secretory granules.

Neuronal characteristics of the neurosecretory cells

Neurosecretory cells appear to be specialized neurons in which the potential capacity for secretory function has become extensively developed and of primary importance. The available evidence indicates that the neurosecretory neurons of the vertebrate neurohypophysis have retained typically neuronal characteristics. It is likely that the neurophysiological and neurochemical events accompanying vasopressin and oxytocin release from neural lobe nerve terminals are analogous to the events mediating release of neurohumoral transmitter substances from non-neurosecretory nerve terminals (Douglas and Poisner, 1964; Yagi, Azuma, and Matsuda, 1966; Mikiten, 1967). It should be noted, however, that information to modify or to control neurosecretory activity might conceivably be directly transmitted (electrically or chemically) from ordinary, non-neurosecretory fibers to the neurosecretory nerve terminals (Bern and Knowles, 1966).

During the past two decades, electrophysiological studies have been made of neurosecretory

systems. Both ordinary nerve fibers and neurosecretory fibers may be found in such nerve tracts (Bern and Knowles, 1966). The recordings of compound action potentials from them have, therefore, established only that some nervous conduction is occurring, not that it is the neurosecretory fibers that are exhibiting nervous conduction. Recent studies with single-unit recording of electrophysiological activity have shown that such activity is circumstantially associated with the release of vasopressin and oxytocin from the neurohypophysis. Thus it has been possible to reach the conclusion that at least some neurosecretory neurons can conduct impulses and that this activity may determine peptide hormone release.

Cross and Green (1959) reported the first unit recordings of electrophysiological activity of neurons of the supraoptic and paraventricular nuclei of the hypothalamus. Cell bodies of neurosecretory fibers of the hypothalamo-hypophysial tract lie within these nuclei (Christ, 1966). Cross and Green (1959) found that the firing frequency of neurons of the supraoptic and paraventricular nuclei in the rabbit tended to be slower than that of neurons in other regions of the hypothalamus, but that the action potential amplitude and wave-form were similar to those of other neurons. They found that some neurons in the

supraoptic and paraventricular nuclei display a remarkable sensitivity to small injections of hypertonic saline solution into the common carotid artery (a stimulus known to cause vasopressin release).

Recordings of electrophysiological activity of neurons of the supraoptic or paraventricular nuclei in the rat and cat have been reported by Suda, Koizumi, and Brooks (1963), Koizumi, Ishikawa, and Brooks (1964), Yagi et al. (1966), Brooks et al. (1966), and Dyball and Koizumi (1969). Dyball and Koizumi (1969) indicated that, in the rat, supraoptic nucleus stimulation seems to be more directly associated with vasopressin release, while paraventricular nucleus stimulation seems more associated with oxytocin release.

Mikiten (1967) studied the electrically stimulated release of vasopressin from the rat neurohypophysis. He obtained evidence that electrical impulses passing down the neurohypophysial stalk are effective in causing release of vasopressin. He noted many similarities between the electrophysiological and chemical factors controlling release of vasopressin from the neural lobe and those controlling release of neurohumoral transmitter substances from non-neurosecretory nerve fibers. Because of these remarkable similarities he suggested that the neurohypophysis might serve as a convenient model system for studying

the mechanisms of release of substances from nerve fibers generally.

THE "CHOLINERGIC LINK" HYPOTHESIS FOR NEURAL LOBE HORMONE RELEASE

The postulate that a cholinergic mechanism might mediate release of vasopressin and oxytocin from the neural lobe was made by Abrahams, Koelle, and Smart (1957). This suggestion was contrary to the conclusion reached earlier by Pickford and her associates (Pickford, 1939, 1947; Duke, Pickford, and Watt, 1950) on the basis of *in vivo* physiological and pharmacological investigations of neural lobe hormone release. Duke *et al.* (1950) had concluded that, although ACh apparently was effective in stimulating release of vasopressin by an action upon the neurosecretory cell bodies, ACh apparently did not mediate release of vasopressin by a direct action upon the terminals of the neurosecretory fibers in the neural lobe.

Abrahams *et al.* (1950) postulated a possible "cholinergic link" for neural lobe hormone release on the basis of their finding that neuron cell bodies in the supraoptic nuclei of the dog stained intensely with a histochemical technique that demonstrates AChE activity. The AChE activity was found only in the region of the cell bodies of these neurons, however. Abrahams *et al.* (1957) could not demonstrate AChE-staining activity for any distance greater than 400

microns down the axons of these neurons. They were unable to detect AChE activity along the hypothalamo-hypophysial tract of the dog. In spite of this lack of AChE activity along the axons, however, Abrahams et al. (1957) suggested that the neurosecretory axons terminating in the neural lobe might be "cholinergic". They suggested that this would involve "a neuron's own transmitter substance providing the stimulus for the release of its own endocrine product."

A subsequent study by Koelle and Geesey (1961) of the neurohypophysis of the cat revealed "moderate" AChE-staining activity along the whole length of the hypothalamo-hypophysial tract. The intensity of AChE-staining of this tract in the cat was said to resemble that of afferent vagal neurons. This was intermediate in intensity between the AChE activity found for "known cholinergic neurons" and "the majority of the adrenergic neurons of the same species." Koelle and Geesey (1961) concluded that their findings supported the postulate by Abrahams et al. (1957) that ACh is liberated from neural lobe nerve terminals, where it brings about the release of oxytocin or vasopressin. Koelle and Geesey (1961) suggested that this local action of ACh would be terminated by hydrolysis by the AChE of axonal terminals.

This hypothesis of Koelle and Geesey (1961),

however, represents only one of the possible interpretations of the presence of "moderate" AChE activity in the hypothalamo-hypophysial tract of the cat. Abrahams et al. (1957) actually suggested an alternative hypothesis in addition to their "cholinergic link" hypothesis. Abrahams et al. (1957) pointed out that strong AChE activity in a neuron cell body is likely to indicate a post-synaptic site that is sensitive to ACh, not a "cholinergic" structure. As Abrahams et al. (1957) pointed out, their finding of no AChE activity along the axons of the hypothalamo-hypophysial tract of the dog supports the view that neurosecretory neurons of that tract are sensitive to ACh but not cholinergic.

Dale (1954) has pointed out an invalid assumption that has sometimes appeared in the discussion of such postulated "cholinergic mechanisms", namely, the "instinctive, though entirely fallacious assumption that cholinergic function implies sensitiveness to acetylcholine." A similar unjustified assumption appears to have been made frequently in discussions of a hypothetical "cholinergic link" for neural lobe hormone release; that is, the assumption that sensitiveness to ACh implies cholinergic function. Dale (1954) mentioned the terms cholinoceptive and adrenoceptive to denote, respectively, sensitiveness to ACh and to the

postganglionic sympathetic catecholamine neurotransmitter. Dale (1954) suggested that the terms cholinoceptive and adrenoceptive on the one hand and cholinergic and adrenergic on the other hand might permit a clear and explicit distinction between sensitiveness to the action of a given transmitter and release of such a transmitter. Such a distinction will be observed in the present study.

More recent experimental observations have suggested that, indeed, the neurosecretory fibers of the mammalian hypothalamo-hypophysial tract are probably cholinoceptive but not cholinergic. Some of this more recent evidence calls into question the interpretation of Koelle and Geesey (1961) that "moderate" AChE activity in the cat hypothalamo-hypophysial tract might indicate cholinergic function for the neurosecretory neurons. Shute and Lewis (1963; 1966a) and Lewis, Shute, and Silver (1964) have studied the AChE-containing and ChAc-containing nerve tracts to the hippocampal formation in the rat brain. Their findings indicate that non-cholinergic nerve fibers may have relatively strong AChE activity in their cell bodies. "The essential histochemical criterion of a cholinergic neuron... appears to be the presence of AChE on the axonal membrane" (Shute and Lewis, 1966a).

Shute and Lewis (1966b) found that the AChE

activity of the supraoptic nucleus of the rat is primarily associated with neuron cell bodies, with no AChE activity demonstrable in the axons of these neurons. They suggested that this pattern of AChE activity was similar to that previously found (Shute and Lewis, 1966a) for other nerve fibers that are evidently not cholinergic.

Gerschenfeld, Tramezzani, and De Robertis (1960) independently suggested essentially the same "cholinergic link" hypothesis as Koelle and Geesey (1961). Gerschenfeld et al. (1960) observed electron microscopically the presence of two apparently "distinct" populations of microvesicles in the nerve terminals of the neurohypophysis of the toad. They described a population of large (1150 Å diameter), dense-core vesicles and a population of smaller (430 Å diameter), clear vesicles. They suggested that the smaller, clear vesicles were so-called "synaptic vesicles" in analogy with the microvesicles supposed to contain ACh in other nerve terminals (De Robertis and Bennett, 1955; De Robertis et al., 1961). They suggested, furthermore, that ACh might be released from these "synaptic vesicles" upon the arrival of an action potential at the neurohypophysial nerve terminal, with the released ACh then mediating some part of the process of secretion of peptide hormones that were supposed to be associated with the larger, granulated "neurosecretory vesicles."

EXPERIMENTAL TESTS OF THE "CHOLINERGIC LINK" HYPOTHESIS FOR NEURAL LOBE HORMONE RELEASE

The hypothesis of a cholinergic mechanism to mediate release of vasopressin and oxytocin from the neural lobe (Gerschenfeld et al., 1960; Koelle and Geesey, 1961) postulated that ACh is stored in the small, clear vesicles (so-called "synaptic vesicles") within neurosecretory nerve terminals. This ACh would then be liberated in response to the electrophysiological activity (action potentials) of these neurosecretory fibers. The liberated ACh would then mediate release of peptide hormone by some local action. The ACh would then be hydrolyzed by AChE of the nerve terminal membrane.

To test this hypothesis Douglas and Poisner (1964) incubated isolated posterior pituitaries of rats in Locke's solution with ACh (10^{-4} g/ml). Experiments were done with and without the cholinesterase inhibitors eserine and di-isopropylphosphofluoridate. In other experiments carbachol (10^{-4} g/ml) was used instead of ACh. None of these treatments increased the release of vasopressin from the isolated posterior pituitaries.

Daniel and Lederis (1966a) studied the effect on vasopressin and oxytocin release of the incubation of isolated posterior pituitary tissue (rat, ox, hedgehog) with ACh (10^{-10} g/ml). Experiments were done with and without eserine. They did not find significant

stimulation of hormone release from isolated posterior pituitaries by this treatment. They indicated that similar results were obtained when higher concentrations of ACh (up to 10^{-6} g/ml) were used. When they incubated the intact hypothalamo-hypophysial tract with ACh in the same manner, however, they found significant stimulation of release of the peptide hormones. They concluded that ACh acts on the neurosecretory neuron cell bodies in the hypothalamus to stimulate release of the peptide hormones, although ACh is ineffective at the level of the nerve terminals in the neural lobe.

Dicker (1966) incubated isolated whole pituitary glands of rats with ACh (10^{-4} g/ml) with and without eserine. He found that the addition of ACh had no effect on the release of vasopressin or oxytocin.

Mikiten (1967) studied the effect of several drugs on the electrically stimulated release of vasopressin from isolated posterior pituitaries of the rat. The drugs studied included a cholinesterase inhibitor (eserine), a muscarinic agonist (pilocarpine), and ACh antagonists (hexamethonium, d-tubocurarine, atropine, and tetraethylammonium (TEA)). No significant effect on the electrically stimulated vasopressin release was observed in the presence of eserine, pilocarpine, hexamethonium, or d-tubocurarine. Atropine had an inhibitory effect on the vasopressin release at 10^{-4} g/ml,

but not at 10^{-5} g/ml. Mikiten (1967) interpreted the inhibitory effect of atropine at 10^{-4} g/ml as probably due to some "non-specific" action of the drug rather than as a specific anti-muscarinic action, since pilocarpine failed to affect the vasopressin release. Mikiten (1967) interpreted a potentiation of vasopressin release observed in the presence of TEA as likely to be accounted for by prolongation of the action potentials. This was postulated to increase the calcium entry into the nerve terminals, resulting in potentiation of vasopressin release.

The evidence provided by several investigators (Douglas and Poisner, 1964; Dicker, 1966; Daniel and Lederis, 1966a; Mikiten, 1967) indicates that, in vitro, neural lobe peptide hormone release is largely uninfluenced by cholinergic agonists, cholinergic antagonists, or anti-cholinesterase drugs. As Mikiten (1967) has pointed out, these findings provide impressive pharmacological evidence against the hypothesis of ACh mediation of peptide hormone release from the neural lobe.

Further indirect evidence against the hypothesis of a cholinergic link for peptide hormone release has been provided by experiments of Dicker and Singh (1969). They found that ACh (10^{-4} g/ml) in the presence of eserine did not bring about depolarization of rat posterior pituitary cells in vitro, although increased

potassium ion concentration did decrease the potential of the cells.

The *in vitro* experiments have provided little or no support for the hypothesis that ACh acts upon the neurosecretory nerve terminals of the neural lobe to mediate peptide hormone release. As Lederis (1967a) has mentioned, however, further evidence from *in vivo* experiments would be of value to confirm the conclusions from the *in vitro* experiments.

INNERVATION OF THE MAMMALIAN PARS NERVOSA

In establishing the significance of ACh in a tissue it is of much importance to understand the distribution within that tissue of nerves that might be cholinergic. As a basis for subsequent discussion of the significance of ACh in the posterior pituitary, the innervation of the neural lobe is described here.

The neurohypophysis consists of three parts which have functional characteristics in common: the median eminence of the tuber cinereum, the infundibular stem, and the infundibular process. The median eminence is a bowl-shaped protruberance located a short distance caudally from the optic chiasma. The infundibular stem is a narrow, tubular structure that emerges from the tuber cinereum and extends obliquely caudad. The infundibular stem terminates in a knob-like enlargement, the infundibular process (synonyms for which are *pars*

nervosa and neural lobe of the pituitary). The neurohypophysis is incompletely enclosed in a sheath of adenohypophysial tissue, composed of pars tuberalis, pars intermedia, and pars distalis (synonym, anterior lobe of the pituitary).

Early work (reviewed by Harris, 1955 and Christ, 1966) established the existence of a large nerve supply to the neural lobe from the hypothalamus. Only since 1938, however, has it become clear that the neurohypophysis closely resembles the central nervous system in structure and function (Bodian, 1966). In 1938 Fisher, Ingram, and Ranson reported their extensive studies of diabetes insipidus induced by neurohypophysial lesions. Their studies brought about a considerable clarification of the relationship of neural lobe innervation to the release of vasopressin and the physiological regulation of water balance. The concept that emerged from the studies of Fisher et al. (1938) was that all levels of the neurohypophysis are functionally under the control of the hypothalamus, most notably the cells of the supraoptic nucleus.

Later work (reviewed by Christ, 1966) has generally confirmed and elaborated the early findings that the main nerve supply of the neurohypophysis originates in the hypothalamus. The hypothalamohypophysial tract, according to the generally accepted

view, consists of the supraoptico-hypophysial tract and the tubero-hypophysial tract. The nerve fibers in these tracts typically are unmyelinated, although myelinated fibers in the tuberal segment are encountered in some species (Christ, 1966).

In addition to the supraoptic nucleus itself, another prominent hypothalamic cell group that contributes to the supraoptico-hypophysial tract is the paraventricular nucleus. The fibers from the paraventricular nucleus run toward the supraoptic nucleus, but their course as a distinct tract is difficult or impossible to trace after they enter the supraoptic nucleus. The precise extent of the contribution of fibers from the paraventricular nucleus to the supraoptico-hypophysial tract has not been determined with certainty (Christ, 1966).

Information on the second part of the hypothalamo-hypophysial tract, the tubero-hypophysial tract, is difficult to evaluate. Although the fibers of the tubero-hypophysial tract apparently have their cell bodies in nuclei in the tuberal region of the hypothalamus, the precise origin of these fibers is difficult to determine. The tubero-hypophysial tract terminates in the median eminence and the proximal part of the infundibular stalk. Typically the fibers terminate either around the capillary loops which run

toward the pituitary portal vessels or on the surface immediately adjacent to the pars tuberalis (Szentágothai et al., 1962).

In the mammalian neurohypophysis, not all of the fibers of the hypothalamo-hypophysial tract come from cells in the supraoptic, paraventricular, and tuberal nuclei. Other fibers from the anterior hypothalamic area, medial forebrain bundle, and periventricular region have been described in the hypothalamo-hypophysial tract of certain species (Green, 1966a).

The supraoptico-hypophysial tract enters the rostral and lateral parts of the median eminence, and the tubero-hypophysial tract enters the caudal part. In the infundibular stalk most of the fibers of the hypothalamo-hypophysial tract congregate into a dense bundle lying in a central position, leaving the peripheral zone close to the pars tuberalis relatively free of neural elements. The diameter of the nerve fibers in the inner layer of the infundibulum varies; both the coarser fibers of the supraoptico-hypophysial tract and some finer fibers can be seen. The finer fibers might possibly be collaterals of the supraoptico-hypophysial tract or fibers of the tubero-hypophysial tract (Christ, 1966).

After the hypothalamo-hypophysial tract

enters the infundibular process the nerve fibers fan out, and the tract becomes a meshwork of fiber bundles. Delicate nerve fibers in the perivascular spaces of the infundibular stem and infundibular process have been demonstrated by many investigators (reviewed by Christ, 1966). Possibly they are autonomic nerve fibers. The heavier fibers that carry histologically demonstrable neurosecretory material do not seem to be impregnated as well with silver stains as the delicate fibers. In the neural lobe the commonest appearance revealed by silver stains is of very delicate swathes of fibers surrounding blood vessels. It is difficult to say whether these may be identified as fine branches from neurosecretory fibers or as nerve fibers of other types (possibly autonomic) (Green, 1966a).

In some species there is a fairly rich innervation of the infundibular stalk and process, as well as of the median eminence, by catecholamine-containing nerve fibers that are revealed by fluorescence microscopy (Björklund, Falck, and Rosengren, 1967; Odake, 1967; Björklund, 1968; Björklund, Enemar, and Falk, 1968). Coarse, varicose fluorescent fibers are mainly localized around larger blood vessels and are at least partly of sympathetic origin. There is in some species (pig, rat, cat, and dog), in addition to these presumably vasomotor fibers, a rich network of

delicate, varicose fibers distributed throughout the parenchyma of the pars nervosa and surrounding the cells of the pars intermedia (Odake, 1967; Björklund, 1968).

Björklund et al. (1967) and Björklund (1968) have indicated that the fluorescence of fibers innervating the pars nervosa of the pig and rat is likely associated with dopamine. Although these fluorescent fibers are apparently not identical with neurosecretory fibers, they appear to be of central rather than autonomic origin.

An important point that should be noted in regard to the patterns of innervation of the mammalian neural lobe is the considerable complexity and species variability of the types of nerve supply that have been observed with light microscopy, fluorescence microscopy, and electron microscopy. There is considerable variation in caliber of the nerve fibers and in the types of microvesicles ("secretory granules" and so-called "synaptic vesicles") to be found within the nerve terminals. The fine-caliber fibers may possibly be autonomic fibers, although this is not certain. There are species differences in the presence or absence of fluorescent monoamine-containing nerve fibers in the neural lobe (Björklund et al., 1968). Different types of fluorescent fibers may be found in the neurohypophysis of a given species (Björklund et al., 1967).

It is well known that there is an autonomic nerve supply to the pars nervosa. Dandy (1913) demonstrated autonomic nerve fibers from the internal carotid plexus entering the neural lobe of the dog along with the median artery supplying that tissue. The autonomic nerve fibers that reach the neural lobe with this artery are distributed with the blood vessels within the tissue. They are closely associated with the vessel walls (Christ, 1966). Although it was assumed by Dandy (1913) that the autonomic nerve fibers reaching the pars nervosa from the internal carotid plexus are sympathetic fibers, later investigations (Cobb and Finesinger, 1932; Chorobski and Penfield, 1932) demonstrated that parasympathetic fibers are distributed to the internal carotid plexus in the monkey. It may be possible, then, that there are cholinergic parasympathetic fibers in the distribution of autonomic nerve fibers to the neural lobe of mammals.

INNERVATION OF THE MAMMALIAN PARS DISTALIS

In contrast to the pars nervosa, which receives an extremely rich nerve supply, the pars distalis possesses a very small nerve supply. It is important to the purpose of the present study to establish the nature and the extent of this nerve supply.

The question of secretomotor innervation

The extent and nature of the nerve supply to

the pars distalis was, in previous decades, the subject of sharp controversy. The resolution of this controversy was vital to an understanding of the nature of the control of the endocrine functions of this tissue. The question was whether such control was effected by secretomotor innervation or whether it was hormonal in the sense that blood-borne chemical substances determined the activity of the anterior lobe.

Serious attention was drawn to the possibility of some type of central nervous system control of anterior lobe endocrine function by early observations (reviewed by Harris, 1948) which indicated that exteroceptive stimuli (visual, olfactory, auditory, and tactile stimuli) in various species exert strong influence on gonadal function. A common characteristic of the effects of such stimuli appeared to be mediation by some nervous mechanism, most probably from the hypothalamus.

By 1948 it was widely accepted that control of the endocrine activity of the anterior lobe was effected by means of humoral transmission through the hypophysial portal blood vessels (Harris, 1948). The hypophysial portal system takes its origin as capillaries in the median eminence of the tuber cinereum; then it forms vascular trunks on the infundibular stalk and breaks up into sinusoids within the pars

distalis. These blood vessels have been found throughout the range of vertebrates. The direction of blood flow in this system has been found to be towards the anterior pituitary (Harris, 1948; Harris, 1955; Harris, Reed, and Fawcett, 1966; Green, 1966a).

Although the evidence is now overwhelming that control of the endocrine function of the anterior pituitary is by blood-borne chemical substances, many investigations were carried out in earlier decades to test the alternative hypothesis that the control of anterior pituitary function might be effected by some secretomotor nerve supply. Since any considerable innervation of the pars distalis by fibers from the hypothalamo-hypophysial tract is generally lacking (Green, 1966a), the investigators who sought a secretomotor nerve supply to the anterior lobe concentrated their attention largely on the autonomic nerve supply. Now that the postulate of secretomotor control of the anterior lobe has been disproved (Harris, 1948; Harris, 1955; Green, 1966a), however, it would appear most probable that the autonomic nerve supply to the anterior lobe is concerned with vascular control, as indicated by Szentágothai et al. (1962).

Autonomic nerves to the mammalian pars distalis

The first detailed description of the autonomic nerve supply to the mammalian pars distalis was

given by Dandy (1913). He found that, in the dog and cat, autonomic nerves from the internal carotid plexus travel along the posterior communicating arteries which supply the hypophysis. From these nerves numerous filaments are given off. They pass along the blood vessels to the infundibular stalk, "from which they delve into the substance of the anterior lobe and are lost to view." Dandy (1913) found that some arterial branches had as many as three or four small filaments, while most had only one or two. All these nerves were said to be "in contact with the sheaths of minute blood vessels."

Hair (1938), in a study of the cat, confirmed Dandy's (1913) description of the autonomic nerve supply of the pars distalis as associated with arteries to that tissue. Hair (1938) found that the number of fibers along a vessel is inconstant, but that it appears to be proportional to the size of the vessel that the fibers accompany. Hair (1938) stated that the course taken by the fibers within the anterior lobe is "extremely tortuous", and that consequently only short segments of them can be traced in a single histological section. He reported that the fibers accompany vascular channels for considerable distances. Then they give off numerous branches that pass between the epithelial cells. He indicated that the terminal parts of the fibers have

less affinity for the silver stain, and that this difference in staining intensity accounted for the low percentage of nerve terminals that he saw in his preparations.

Drager (1944, 1945) found that the majority of nerve fibers in the anterior lobe of man, ox, cat, dog, mouse, rat, and rabbit are of autonomic origin. Such fibers are more numerous at the periphery of the gland. They are found in close association with blood vessels. Drager (1945) said that, in view of the successful demonstration of numerous nerve fibers in various structures adjacent to the pars distalis, it is unlikely that the failure to find numerous nerve fibers in the anterior lobe could be attributed to faulty histological technique.

Rasmussen (1938) made a careful study of the innervation of the human hypophysis, using the fresh hypophyses of animals (rat, guinea-pig, cat, dog, and monkey) as a check on what was lost by postmortem changes in the human tissue. He found that a number of small nerves composed of unmyelinated fibers are present in the connective tissue capsule of the pituitary. These fibers (on the order of 200 in the human) are of autonomic origin and are found following the course of blood vessels. They were found to be most abundant on the upper surface of the anterior lobe. A considerable number of these fibers pass into the anterior lobe,

where they spread out anteriorly and laterally with the connective tissue and blood vessels of the upper superficial region. The fibers become more and more scattered, but individual fibers may be seen in the vascular stroma between cords of cells.

Rasmussen (1938) found also a prominent bundle of as many as 50 fibers commencing just lateral to the infundibulum on either side and frequently descending for a considerable distance into the interior of the anterior lobe. This bundle of fibers usually became associated with an artery and several prominent veins. Some of these fibers disseminated themselves among the gland cells deep in the anterior lobe. Rasmussen (1938) found a few nerve fibers passing from the neural lobe through the region of the pars intermedia and on into the pars distalis for a slight distance, "but the number is negligible." In view of the autonomic origin of most nerve fibers in the pars distalis, Rasmussen (1938) concluded that the nerve fibers in the anterior lobe are "most likely connected with the vascular system."

The innervation of the bovine pars distalis was studied by Gilmore, Petersen, and Rasmussen (1941). Although they found nerves in the pars intermedia, no nerves were found entering the pars distalis from the pars intermedia. Gilmore et al. (1941) did not report extensive innervation of the bovine pars distalis.

Ribas-Mujal (1958) also has studied the innervation of the bovine pars distalis. He found a considerable number of unmyelinated nerve fibers in the pars intermedia, but very few of these were found to run toward the pars distalis. He found only a few scattered nerve fibers in the bovine pars distalis.

Recently the fluorescence microscopy technique has been employed in studies of the monoamine-containing cells and nerve fibers found in the mammalian pars distalis (Dahlström and Fuxe, 1966; Odake, 1967; Björklund et al., 1967). Dahlström and Fuxe (1966) found fluorescence attributable to primary catecholamines in cells of the adenohypophysis of the mouse, rat, guinea-pig, rabbit, cat, dog, and monkey. They found a plexus of catecholamine-containing nerve terminals of central origin in the pars intermedia in all the species studied, but they did not report passage of such fibers into the pars distalis. They reported only that, in the cat, a very few adrenergic nerve terminals were observed in the pars distalis.

Odake (1967) found, in the dog and cat, numerous catecholamine-containing fluorescent nerve fibers in the pars nervosa, but he reported that no fluorescent varicose fibers from the neurohypophysis normally penetrated into the pars distalis.

Björklund et al. (1967) performed a combined

chemical and histochemical study of monoamines in the hypophysis of the pig. They found that there were very few fluorescent nerve fibers in the pars distalis, although some of the gland cells of the pars distalis show histochemical fluorescence.

Generally all the recent evidence from adequately controlled histological studies of the mammalian pars distalis indicates that the nerve supply of that tissue consists almost entirely of a small autonomic nerve supply (Szentágothai et al., 1962). In the absence of any considerable adrenergic nerve supply as demonstrated by the fluorescence microscopy technique, it appears likely that a considerable proportion of the small autonomic nerve supply to the mammalian pars distalis is cholinergic.

The possibility of parasympathetic nerve supply to the mammalian pars distalis

Cobb and Finesinger (1932) and Chorobski and Penfield (1932) described a parasympathetic innervation of the arteries of the pia mater in the monkey. Chorobski and Penfield (1932) interpreted their findings to indicate that, in the monkey, parasympathetic innervation derived from the seventh cranial nerve joins the internal carotid plexus as a branch from the greater superficial petrosal nerve.

Hinsey and Markee (1933) suggested that para-

sympathetic fibers carried along with the internal carotid plexus might reach the mammalian pars distalis. Zacharias (1941) described, in the rat, nerve branches from the junction of the greater superficial petrosal nerve and the deep petrosal nerve to the internal carotid plexus and the capsule of the anterior lobe of the pituitary.

Hair and Mezen (1939) and Vogt (1942) studied the possible functional significance of the postulated parasympathetic innervation of the rabbit pars distalis. In both series of experiments it was found that removal of the greater superficial petrosal nerves apparently had little effect on the endocrine function of the pars distalis. Other investigations (reviewed by Harris, 1948; 1955) generally failed to provide convincing evidence that a parasympathetic nerve supply to the mammalian pars distalis might have an important physiological role to play in the control of the endocrine function of that tissue. On the other hand, however, no evidence was provided to disprove the postulate that there is a parasympathetic nerve supply to the pars distalis.

INNERVATION OF THE MAMMALIAN PINEAL BODY

The questions related to the physiology of the mammalian pineal body (synonym: epiphysis cerebri) are complex, but there is now some general agreement that

the mammalian pineal body is an endocrine tissue whose parenchymal cells produce and possibly store chemical compounds which are secreted into the rich pineal vascular system (Kappers, 1965). The information available on the physiological function of the mammalian pineal body has recently been reviewed in the monograph of Wurtman, Axelrod, and Kelly (1968).

It has long been known that nerve fibers from both the habenular commissure and the caudal commissure are found in the habenula that is attached to the pineal body. The mammalian pineal body shows an intimate topographical relationship with these commissures from its early developmental stages (Kappers, 1965).

In addition to these fibers of cerebral origin (from the habenular and caudal commissures), autonomic nerve fibers to the pineal body have been observed by a number of authors. Opinions have differed, however, regarding the origin, the course, the site of termination, and the functional significance of the cerebral as well as the autonomic fibers. Observations relevant to these questions about the innervation of the pineal body have been reviewed by Kappers (1960, 1965) and Wurtman *et al.* (1968).

Cerebral fibers to the mammalian pineal body

In a study of the pineal body of the monkey, Le Gros Clark (1940) found that nerve fibers from the

habenular commissure enter the pineal stalk, but that they arch and leave the pineal body again, rejoining the habenular commissure on the other side. Le Gros Clark (1940) interpreted this observation to mean that these fibers do not actually innervate the pineal parenchymal tissue. He described them as "merely aberrant commissural fibers."

Le Gros Clark (1940) found that the distribution of fibers to the pineal body from the posterior commissure was essentially the same as that which he described for fibers from the habenular commissure, with one exception: a few fibers from the posterior commissure leave the aberrant fasciculi and ramify immediately beneath the ependymal lining of the posterior wall of the pineal recess. Le Gros Clark (1940) indicated that these fibers have the appearance of terminal fibers, although they do not actually innervate parenchymal cells. Kappers (1965) indicated complete agreement with Le Gros Clark (1940) in supposing that (at least in the macaque monkey, ox, sheep, dog, cat, and rat) most if not all nerve fibers entering the pineal by way of the habenular and posterior commissures are "aberrant fibers."

Autonomic fibers to the mammalian pineal body

The mammalian pineal body receives an abundant supply of autonomic nerve fibers. Early investigations

of this autonomic nerve supply have been reviewed by Kappers (1960).

Phylogenetically the separation of the pineal body from the brain accompanied other fundamental changes in pineal anatomy and function. Rather than photoreceptor organelles, mammalian pineal cells have become specialized as pinealocytes that are evidently concerned with synthesis and release of hormones. Instead of a direct link to the brain, the mammalian pineal body receives innervation by autonomic fibers from two sources, the nervi conarii and the fibers accompanying blood vessels. Within the pineal, many autonomic nerve fibers terminate among the pinealocytes "and thereby provide an indirect path for the control of pineal function by the central nervous system" (Wurtman et al., 1968). The concept that autonomic innervation of the pineal body exerts control over the physiological functions of that tissue has been discussed in detail by Wurtman et al. (1968). The autonomic innervation of the pineal body will be described here to provide a basis for assessing the possibility of cholinergic innervation of the pineal body. No attempt will be made here to resolve the complex questions about the precise physiological significance of this innervation as discussed by Kappers (1965), Wurtman et al. (1968), and other authors.

Kappers (1960) has indicated that, in many

instances, the nerve bundles seen running along blood vessels to the pineal body do not surround the vessels and do not form vascular plexuses. Kappers (1960) suggested that these nerve fibers might be following the vessels merely as a means of entry into the pineal body, and that such fibers may not be concerned primarily with vasomotor control.

Kappers (1960) found that by far the largest supply of autonomic fibers to the pineal body of the rat is by way of nerve bundles entering the pineal at its dorsolateral surface (at the opposite pole of the pineal body from its stalk). Le Gros Clark (1940) described a single nerve corresponding to this description in the monkey ("nervus conarii"), but Kappers (1960) observed paired nerves in the rat ("nervi conarii"). Kappers (1960, 1965) suggested that the existence of a single such nerve in some animals and paired nerves in others may be related to the topographical position of the tentorium cerebelli in relation to the tip of the pineal, and thus to the distance allowed for unpaired nerves to join.

From the point of entry into the pineal body of the monkey, the nervus conarii pursues an unbranched course to the dura mater of the wall of the straight sinus (Le Gros Clark, 1940). Le Gros Clark (1940) found that individual fibers do not leave the main fasciculus to

enter surrounding tissue. Kappers (1960) found that, in the albino rat, the nervi conarii often enter the pineal body at the point of entry of the main vascular supply to that tissue (branches of the posterior cerebral artery).

There seems to be general agreement on the course of the nervi conarii, but the actual origin of the fibers of these nerves is the topic of controversy. It appears that there may be some variation from one species to another in the origin of the nervi conarii.

Kappers (1960, 1965) indicated that the origin of the fibers of the nervi conarii in the albino rat is from the superior cervical sympathetic system. Other authors have agreed with this conclusion (Wurtman et al., 1968). The fact remains, however, that numerous fibers in the nervus conarii of the monkey were found by Kenny (1961) to degenerate after the greater superficial petrosal nerve was cut, indicating a parasympathetic origin for such fibers. Kenny's (1961) experimental observations support the suggestion made by Le Gros Clark (1940) that parasympathetic innervation of the pineal might exist in the monkey. Le Gros Clark (1940) noted that, in the monkey, some fibers of the nervus conarii are rather heavily myelinated. Le Gros Clark (1940) suggested that such heavily myelinated fibers are not likely to be postganglionic sympathetic fibers

derived from the cervical sympathetic ganglia.

Cobb and Finesinger (1932) and Chorobski and Penfield (1932) have described parasympathetic nerve fibers following the course of cerebral blood vessels in the monkey. Some of these fibers were found to be myelinated. This observation appears to give some support to the suggestion of Kenny (1961) that the nervus conarii may carry parasympathetic fibers in the monkey. Kappers (1965) and Wurtman et al. (1968) have remarked that, in view of the observations reported by Kappers (1960) and Kenny (1961), the possibility of species variability exists in regard to extent of parasympathetic innervation of the pineal.

Owman (1965) has studied the monoaminergic innervation of the mammalian pineal body by the fluorescence microscopy technique. He interpreted his observations to indicate that in some species a cholinergic innervation of the pineal may have relatively more significance than in other species. He observed that the adrenergic innervation of the porcine and bovine pineal is particularly scarce. Owman (1965) cited the histochemical demonstration of cholinesterase (ChE) activity in these tissues (Arvy, 1965) as an indication that the scarcity of adrenergic innervation of the pineal of the pig and ox might be made up for by a relatively greater cholinergic innervation in these species.

Arvy (1965) reported that the AChE activity in the pineal body of the pig, sheep, and ox is primarily confined to fine nerve fibers that accompany larger blood vessels in the postero-ventral portion of the tissue. She remarked that AChE-staining nerve fibers were relatively scarce in the antero-dorsal as compared with the postero-ventral portion of the pineal body. The only other AChE-containing fibers that she described were scattered myelinated fibers passing into and back out of the pineal body in the habenular and posterior commissures. Arvy (1965) remarked, however, that the AChE-containing fibers in these commissures hardly penetrate past the surface of the pineal body into the tissue.

In view of the findings of Owman (1965) and Arvy (1965) on the relative extent of adrenergic nerve fibers and AChE-containing nerve fibers to the bovine pineal, it appears likely that a considerable proportion of the nerve supply to the bovine pineal may be cholinergic. Further consideration of this possibility will be given in the discussion of the ACh content found for the bovine pineal body in the present study.

Other nerve fibers of the mammalian pineal body

Le Gros Clark (1940) found numerous cells believed to be nerve cell bodies in the center of the pineal body of the monkey. He reported that these cells were relatively large, that they were usually

multipolar (but sometimes bipolar), and that they gave rise to numerous branching processes. They were found located mainly in the center of the distal half of the pineal body, where they were embedded in a mass of neuropil. Le Gros Clark (1940) reported finding occasional cells of a similar type in the proximal half of the pineal body. He noted that such cells had been described previously by others, but he concluded that only in the monkey had such nerve cells within the pineal been identified with certainty.

Kappers (1965) has pointed out that, if parasympathetic nerve fibers do indeed reach the pineal body of the monkey via the nervi conarii, then the nerve cell bodies within the pineal of the monkey (Le Gros Clark, 1940) would probably be parasympathetic ganglion cells. Kappers (1965) stated, in agreement with Le Gros Clark (1940), that in non-primate mammals such nerve cell bodies within the pineal do not occur regularly.

In the mammalian pineal body, nerve fibers originating in that tissue and running to the brain are absent. In this regard the nerve supply of the mammalian pineal body is in sharp contrast to that of the pineal body and the accessory pineal organ of non-mammalian vertebrates. In these non-mammalian tissues the only nerve fibers clearly demonstrated so far are afferent fibers with cell bodies in the pineal tissue (Kappers, 1965).

II. MATERIALS AND METHODS

MATERIALS

Bovine pituitary glands, brain, and pineal bodies were obtained from animals of either sex and various ages from Canada Packers, Ltd. of St. Boniface, Manitoba. The bovine tissues, contained in vessels surrounded by ice, arrived at the laboratory about one hour after the death of the animals. Dissection of bovine posterior pituitary, anterior pituitary, hypothalamus, and pineal body to free these tissues from surrounding tissue was done in a cold room at 4^{o} C.

Whole brains (the cerebrum, severed from the brainstem at the collicular level) of 200-400 g rats (from our own hooded colony of random-bred Wistar derivative rats) were removed immediately after decapitation. Decapitation of the rats and dissection of the brain tissue were done in the cold room at 4^{o} C. Homogenization of the rat brain tissue was completed within a few minutes of the death of the animals.

Tissue homogenization was done with tissue grinders obtained from the Kontes Glass Company, Vineland, New Jersey. The close-fitting, ground-glass grinding surfaces of the glass pestle and the glass receiver were tapered conically from the cylindrical upper portion of the tissue grinder. The tissue grinder pestle shaft was driven by an electric motor at 1000-2000 revolutions per minute. All tissue homogenization was carried out in the

4° cold room with the tissue grinder held in a beaker full of crushed ice.

Reagents of analytically pure grade were obtained from commercial sources unless otherwise specified. Acetylcholine (ACh) chloride, propionylcholine (PrCh) chloride, butyrylcholine (BuCh) chloride, eserine sulfate, and bovine erythrocyte acetylcholinesterase (AChE) were obtained from Sigma Chemical Company, St. Louis, Missouri. Choline (Ch) chloride, atropine sulfate, ammonium reineckate, chloroplatinic acid, potassium iodide, ammonium acetate, glacial acetic acid, and toluene (sulfur-free) were obtained from British Drug Houses, Toronto, Ontario.

The ion-exchange resins Dowex 1-X8 (100-200 mesh; chloride form) and Bio-Rex 70 (100-200 mesh; sodium form) were obtained from Bio-Rad Laboratories, Richmond, California. Ethylene glycol monomethyl ether, isopropyl alcohol, and 1,4-dioxane were obtained from Fisher Scientific Company, Don Mills, Ontario. Naphthalene (recrystallized from alcohol) was obtained from Eastman Organic Chemicals, Rochester, New York. 2,5-Diphenyloxazole (PPO) was obtained from Nuclear Enterprises, San Carlos, California. 1,4-Bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl-POPOP) was obtained from Packard Instrument Company, Downers Grove, Illinois. Acetyl-1-^{14}C-choline iodide and choline-methyl-^{14}C chloride were obtained from New England Nuclear Corporation, Boston, Massachusetts. Heparin sodium U.S.P. (Code:HOST) was obtained from Connaught Medical Research Laboratories, Toronto, Ontario.

Scintillation counting was done at 4° C with a Packard TriCarb or a Philips Liquid Scintillation Analyzer. The scintillation medium was of the following composition: one l toluene, one l 1,4-dioxane, one l ethylene glycol monomethyl ether, 240 g naphthalene, 15 g PPO, and 1.5 g dimethyl-POPOP.

EXTRACTION OF ACh FROM TISSUES

Several extraction procedures were used. In each case the procedure to extract the total ACh content of the tissue involved dissection to remove surrounding tissue, mincing of the tissue with scissors under an appropriate small volume of extracting medium at 0° C, and homogenization with the tissue grinder. Centrifugation of the homogenate at 10,800 g X 20 min was followed by washing the pellet twice with the appropriate extraction medium before the supernatant and washes were lyophilized. All homogenization and centrifugation procedures were carried out at 0-4° C. ^{14}C-ACh or unlabeled ACh was routinely added to a sample of the homogenate in most of the experiments. This was done for verification of the identity of behavior of endogenous ACh and added ACh in the chemical purification procedures and for obtaining quantitative estimates of ACh recovery.

Extraction by hydrochloric acid-eserine solution was used together with the reineckate precipitation procedure for ACh as described by Aprison and Nathan (1957)

in some preliminary experiments. The tissue was homogenized in a 2 X 10^{-4} M eserine solution of pH 1.8 (2 ml per g of tissue). After adjustment of the pH to 4.0 the homogenate was held in a boiling water bath for 10 min, then cooled to 0° C and centrifuged. The precipitate was washed with 3 ml water. The combined supernatant and wash was lyophilized, and the residue was taken up in the appropriate volume of Tyrode solution for precipitation with ammonium reineckate.

The perchloric acid extraction procedure described by Morris et al. (1965) was used in some experiments, acetic acid-ethanol extraction (Crossland, 1961) in others, and formic acid-acetone (Toru and Aprison, 1966) in still others.

Extracts were lyophilized under oil pump vacuum with a dry ice-isopropyl alcohol vapor trap.

ANION-EXCHANGE COLUMN CHROMATOGRAPHY

The tissue samples to be carried through the extraction and purification procedures were divided into one-gram portions so that extract corresponding to no more than approximately one g of fresh tissue was chromatographed on any one ion-exchange column.

In most experiments each tissue extract sample was routinely passed through a 0.6 cm X 10 cm column of Dowex 1-X8 anion-exchange resin. The resin was prepared for use by removal of fines (repeated sedimentation and

decantation of the water) and washing with at least one 1 of 1.0 N HCl followed by washing with at least one 1 of distilled-deionized water. After preparation of the columns they were washed with deionized water until the pH of the effluent was 5.0 or higher.

The anion-exchange chromatography of tissue extracts was performed routinely prior to the other purification procedures (paper electrophoresis or cation-exchange chromatography) in order to eliminate polyvalent anions, which were found in control experiments to interfere with the electrophoretic mobility and cation-exchange column retention characteristics of ACh. Poor and variable results with paper chromatographic isolation of ACh due to the presence of polyvalent anions has been described by Whittaker (1963). The necessity of removing interfering anions by anion-exchange chromatography prior to some bioassay procedures has been pointed out by Hanin and Jenden (1966).

After removal of all the extraction solvent by lyophilization the residue of each sample was taken up in a small volume (1-2 ml) of the appropriate solvent and put onto a Dowex 1-X8 column. For anion-exchange chromatography of tissue extract reineckates the eluting solvent was 25 ml 50/50 (v/v) acetone-water. Where the ACh of tissue extracts was to be purified by paper electrophoresis, the Dowex 1-X8 eluting solvent routinely used

was 10 ml 70/30 (v/v) methanol-water. Where the ACh of tissue extracts was to be isolated by cation-exchange chromatography, the Dowex 1-X8 eluting solvent was 10 ml 50/50 (v/v) acetone-water.

After Dowex 1-X8 chromatography and evaporation of the eluting solvent *in vacuo* the residue of each sample was taken up in a small volume of water (0.5-1.0 ml) prior to the paper electrophoresis or cation-exchange chromatography procedures.

CATION-EXCHANGE COLUMN CHROMATOGRAPHY

Isolation of ACh by cation-exchange chromatography was performed as described by Whittaker (1963). Removal of fines and regeneration of the Bio-Rex 70 resin in the hydrogen form by treatment with 1.0 N HCl and water were done by the same operations already described for the preparation of Dowex 1-X8 resin. The final step in preparation of the Bio-Rex 70 columns was elution of the columns with 0.1 M NaH_2PO_4 until the pH of the effluent was equal to the pH of the buffer going onto the column.

Tissue extract samples (equivalent to one g of fresh tissue per column) were fractionated on 0.6 cm X 12.0 cm columns of Bio-Rex 70 with 0.1 M NaH_2PO_4 as the eluting solvent. Two-ml fractions were collected.

The identity of ACh or a closely related choline ester in the tissue extracts was confirmed by chromatography

of ^{14}C-ACh added to a representative sample of each tissue extract. Aliquots were taken for scintillation counting from the fractions from chromatography of tissue extract samples with added ^{14}C-ACh. The fractions taken for guinea-pig ileum bioassay of endogenous ACh of a tissue extract corresponded to the fractions found to contain appreciable radioactivity where ^{14}C-ACh had been added to another sample of the same extract.

Duplicate aliquots were taken from the Bio-Rex 70 chromatography fractions containing added ^{14}C-ACh for calculation of the recovery of ACh added at the time of homogenization. These aliquots had been carried through the same extraction and purification procedures that were applied to samples taken for bioassay of endogenous ACh activity.

Except in a few preliminary experiments where the ACh activity of every individual fraction was estimated by bioassay, the bioassay of the ACh activity of tissue extracts purified by cation-exchange chromatography was carried out after combination of all the ACh-containing fractions into a single sample. After the appropriate fractions were combined and lyophilized the residue from each sample was extracted with several small portions (0.5-2.0 ml) of methanol. After centrifugation the methanol was evaporated from the supernatant, and this final residue was taken up in 1.0 ml water. The final purified

sample was stored frozen until bioassay was performed. The extraction of the lyophilized Bio-Rex 70 fractions with methanol was done to separate the ACh from the NaH_2PO_4 residue (Gardiner and Whittaker, 1954).

PAPER ELECTROPHORESIS

High-voltage paper electrophoresis was performed at 4000 V (80 V/cm) for 20 min at room temperature with a Camag high-voltage electrophoresis system 60510. Whatman 3MM paper strips (39.8 cm X 20.2 cm) were wetted with buffer (0.02 M ammonium acetate brought to pH 4.0 by addition of glacial acetic acid) prior to application of sample. No more than 25 µl of tissue extract was applied over 2.5 cm of origin (500 µl of extract corresponding to about one g of fresh tissue). An origin of 12.7 cm length was used on each paper for tissue extract electrophoresis, and a separate 2.5-cm zone close to the border of each paper was used for electrophoresis of a sample of ^{14}C-ACh (2 µl of 10^{-3} $\mu C_i/\mu l$). In each experiment an appropriate aliquot of the tissue extract with added ^{14}C-ACh was used to verify the position of ACh on the electrophoresis paper. The electrophoresis papers were dried in room air.

Where unlabeled Ch and Ch esters were subjected to paper electrophoresis with or without added tissue extract, the dried electrophoresis papers were sprayed with potassium iodoplatinate reagent prepared according

to the procedure given by Mannering et al. (1954) for visualization of these compounds. Where ^{14}C-Ch and ^{14}C-ACh were subjected to paper electrophoresis with or without added tissue extract, 2.5-cm strips were cut from the dried paper along the path of migration from origin to cathode. Localization of radioactivity on these paper strips was determined by scanning them with a Nuclear-Chicago Actigraph III, Model 10002.

The ACh-containing portion of each electrophoresis paper was cut into small pieces and extracted several times with 50/50 (v/v) methanol-water (25-50 ml per wash). After evaporation of the combined washings in vacuo the residue (usually the combined material from two electrophoresis runs) was taken up in 1.0 ml water. In the case of extracts containing added ^{14}C-ACh, aliquots were taken from this final purified sample for scintillation counting. Where guinea-pig ileum bioassay of the final purified sample was to be done, the sample was stored frozen until the time of bioassay.

RECOVERY EXPERIMENTS

At the time of homogenization each tissue was divided into equal portions of approximately one g each. After homogenization 50 µl of a solution containing ^{14}C-ACh (10^{-3} $\mu C_i/\mu l$) was added to one of these samples. The samples with added ^{14}C-ACh were carried through the same extraction procedures as samples to which no ACh had been added.

Scintillation counting of the radioactivity in aliquots of the purified extracts with added ^{14}C-ACh permitted estimates to be made of the losses of added ^{14}C-ACh at various stages of the extraction and purification procedures. Verification of the recovery estimates using added ^{14}C-ACh was made by adding unlabeled ACh at the same point (immediately after homogenization) in a few experiments and carrying the samples with added unlabeled ACh through the same procedures. In other experiments the recovery of unlabeled ACh alone was determined. The biological activity of added unlabeled ACh alone or added to tissue extracts was estimated by the same bioassay preparations used to estimate the concentration of endogenous ACh in the tissue extracts. Calculation of the tissue concentrations of ACh included a factor based on the recovery of added ACh.

BIOASSAY OF ACh.

Extraction of ACh from tissue samples was always followed by some chemical purification procedure prior to bioassay. Bioassay was done with the isolated guinea-pig ileum as described by Aprison and Nathan (1957). The effectiveness of the various purification procedures is discussed in the Results section.

The bioassay of purified tissue extracts was routinely carried out on more than one section of isolated guinea-pig ileum on the same day, followed on a subsequent

day by repetition of this procedure with different ileum preparations (if there was sufficiently high ACh activity in the extract to permit this experimental design). The bioassay data are presented in the Results section in such a way as to indicate the extent of agreement of bioassay estimations of tissue ACh concentration in terms of reproducibility.

Tissue extract ACh concentrations were determined from the line obtained by linear regression (Steel and Torrie, 1960) of guinea-pig ileum responses (mm contraction) on the logarithm of the concentrations of standard ACh. All tissue extract responses were within the range of the standard curve.

The identity of ACh in tissue extracts was substantiated by testing for complete blockade of the guinea-pig ileum stimulating activity by incubation of the ileum section with 2.9×10^{-8} M atropine for 2 min immediately prior to administration of the purified tissue extract sample. This concentration of atropine was found in control experiments to be just above the minimum concentration required for complete blockade of the isolated guinea-pig ileum contraction due to ACh, but it is well below the concentration required for the complete blockade of the response to histamine. The use of this low concentration of atropine to distinguish isolated guinea-pig ileum activity caused by ACh from the activity caused by histamine

has been described by Gaddum and Picarelli (1957) and by Cheema (1965).

INACTIVATION STUDIES ON TISSUE EXTRACTS

Some aliquots of tissue extracts were held at 98° C at pH 4, and others at pH 10. Boiling of ACh solutions at pH 4 does not lead to rapid destruction of ACh, but boiling at pH 10 leads to complete hydrolysis of ACh in a few min (Whittaker, 1963). In these experiments the acidified and boiled samples were brought to pH 7 prior to bioassay.

A sample of each purified tissue extract was incubated with bovine erythrocyte AChE at 37° C for 15 min, brought to pH 4 by addition of dilute perchloric acid, held at 98° C for 5 min, and brought back to pH 7 by addition of dilute NaOH. Appropriate blanks were prepared.

III. RESULTS

EXTRACTION AND ISOLATION OF ACh FROM TISSUES

The reineckate precipitation procedure for ACh in tissue extracts (Aprison and Nathan, 1957) was applied to HCl-eserine extracts of rat brain and bovine posterior pituitary in preliminary experiments. The rat brain extract reineckate appeared to be fairly free of biologically active interfering material, but the posterior pituitary extract reineckate was badly contaminated with biologically active material that interfered with the guinea-pig ileum bioassay for ACh. Table 1 shows that the ileum stimulating activity of posterior pituitary extract reineckates was only about half blocked by concentrations of atropine that blocked most or all of the activity of rat brain extract reineckates. The reineckate precipitation procedure was eliminated from further consideration in this study.

Perchloric acid, acetic acid-ethanol, and formic acid-acetone extraction procedures were used in the present study. Paper electrophoresis and cation-exchange chromatography were used for isolation of ACh from materials that would interfere with the guinea-pig ileum bioassay for ACh.

PAPER ELECTROPHORESIS

High-voltage paper electrophoresis gave clear-cut separation of unlabeled choline (Ch), ACh, propionylcholine (PrCh), and butyrylcholine (BuCh) (Fig. 1).

TABLE 1

GUINEA-PIG ILEUM BIOASSAY FOR ACh IN REINECKATE PRECIPITATES

Material	Experiment	[Atropine] ($Mx10^{-8}$)	Contraction (mm)
PP	1	0.0	13
reineckate		14.4	6
	2	0.0	46
		14.4	28
	3	0.0	50
		8.6	32
RB	4	0.0	10
reineckate		14.4	0
	5	0.0	13
		8.6	2
Std. ACh	6	0.0	76
reineckate		2.9	6
Std. ACh	7	0.0	91
solution		2.9	3
Std. ACh	8	0.0	56
solution		2.9	0

PP = bovine posterior pituitary; RB = rat brain (cerebrum).

Figure 1

Resolution by paper electrophoresis of Ch and Ch esters. Ten μl of each compound indicated was applied at the origin. After electrophoresis the strips were dried and sprayed with iodoplatinate for visualization of the compounds. The following abbreviations are used: Ch, choline; ACh, acetylcholine; PrCh, propionylcholine; BuCh, butyrylcholine.

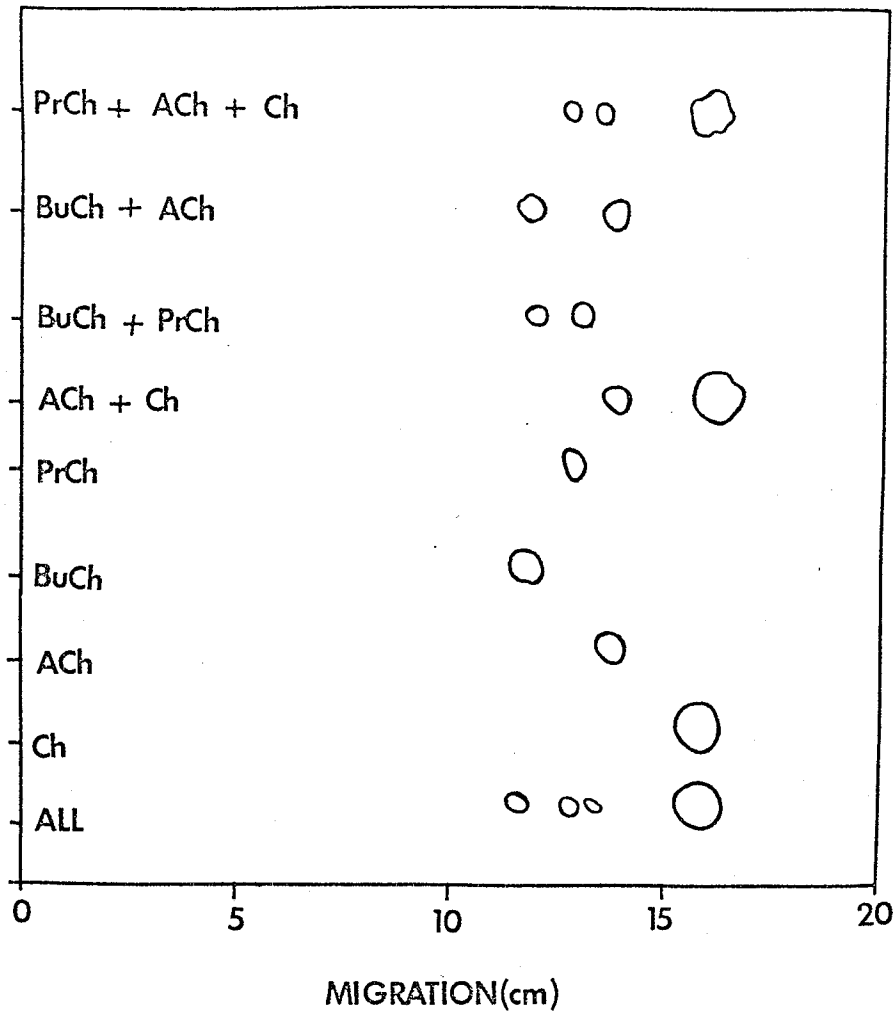

PrCh + ACh + Ch
BuCh + ACh
BuCh + PrCh
ACh + Ch
PrCh
BuCh
ACh
Ch
ALL
0
5
10
15
20
MIGRATION(cm)

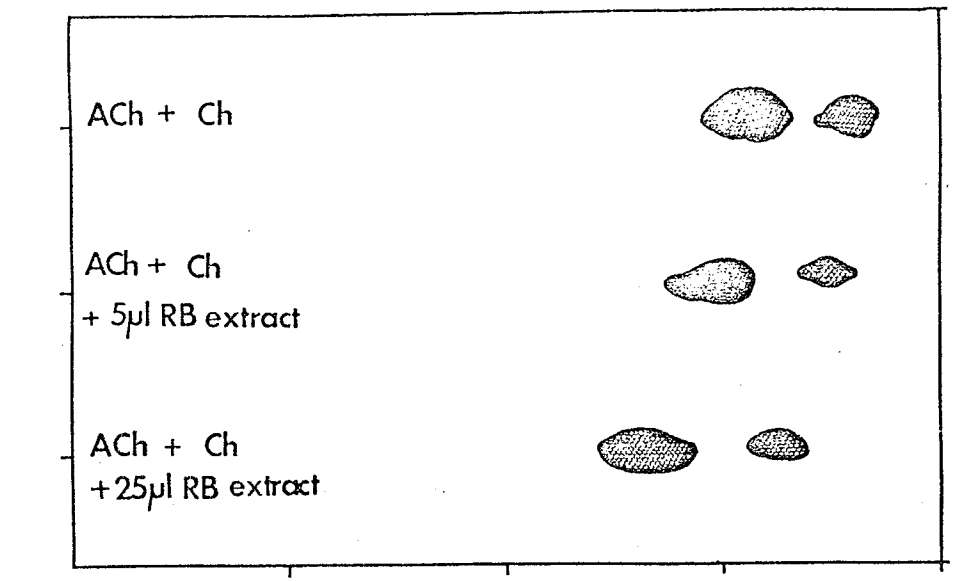
Fig. 2 shows that the electrophoretic behavior of unlabeled Ch and ACh is identical to the electrophoretic behavior of ^{14}C-labeled Ch and ACh when the unlabeled compounds and the labeled compounds are added to identical quantities of tissue extract and subjected to identical electrophoresis conditions. Fig. 2 demonstrates that, although the electrophoretic mobilities of Ch and ACh are decreased in the presence of tissue extract, their mobilities relative to each other remain essentially unchanged.

Preliminary experiments with paper electrophoresis of formic acid-acetone tissue extracts showed that the electrophoretic resolution of ^{14}C-ACh in such extracts was much improved by repeated treatment with warm ethanol and rejection of the resulting precipitates. This improvement in resolution was probably due to the removal of extraneous salts from the tissue extracts. Removal of salts from tissue extracts by treatment with organic solvents has recently been described by Bull, Hebb, and Morris (1969).

The paper electrophoresis characteristics of perchloric acid tissue extracts were much inferior to those of formic acid-acetone tissue extracts. Fig. 3 shows the electrophoretic migration patterns found for ^{14}C-ACh added to perchloric acid extracts and to formic acid-acetone extracts of rat brain and bovine posterior

Figure 2

Resolution by paper electrophoresis of Ch and ACh. Ten µl each of unlabeled choline (Ch) and acetylcholine (ACh) was subjected to electrophoresis with and without added rat brain (RB) extract. ^{14}C-Ch and ^{14}C-ACh (10^{-3} µCi of each) was added to 25 µl of RB extract (corresponding to 60 mg fresh tissue) prior to electrophoresis. The 5-µl aliquot of RB extract corresponded to 12 mg fresh tissue. The RB extract was chromatographed on Bio-Rex 70 and Dowex 1 ion-exchange resins prior to electrophoresis. Unlabeled Ch and ACh were visualized after spraying with iodoplatinate. Radioactivity (counts per min = CPM) was detected by paper strip scanning.


ACh + Ch
ACh + Ch
+ 5µl RB extract
ACh + Ch
+25µl RB extract

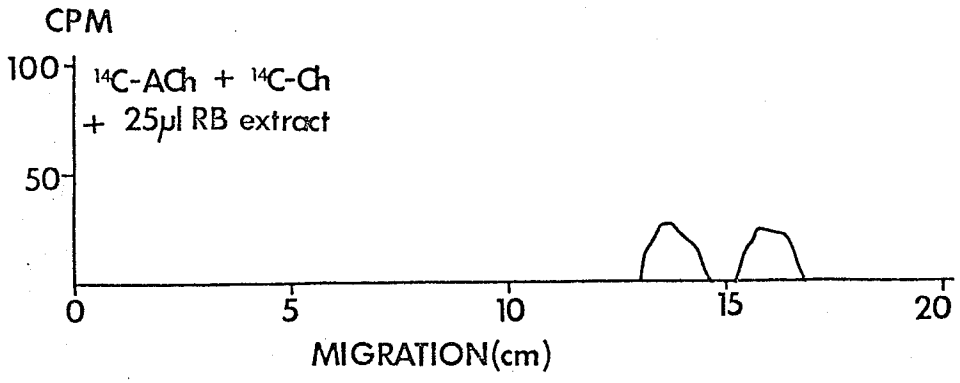
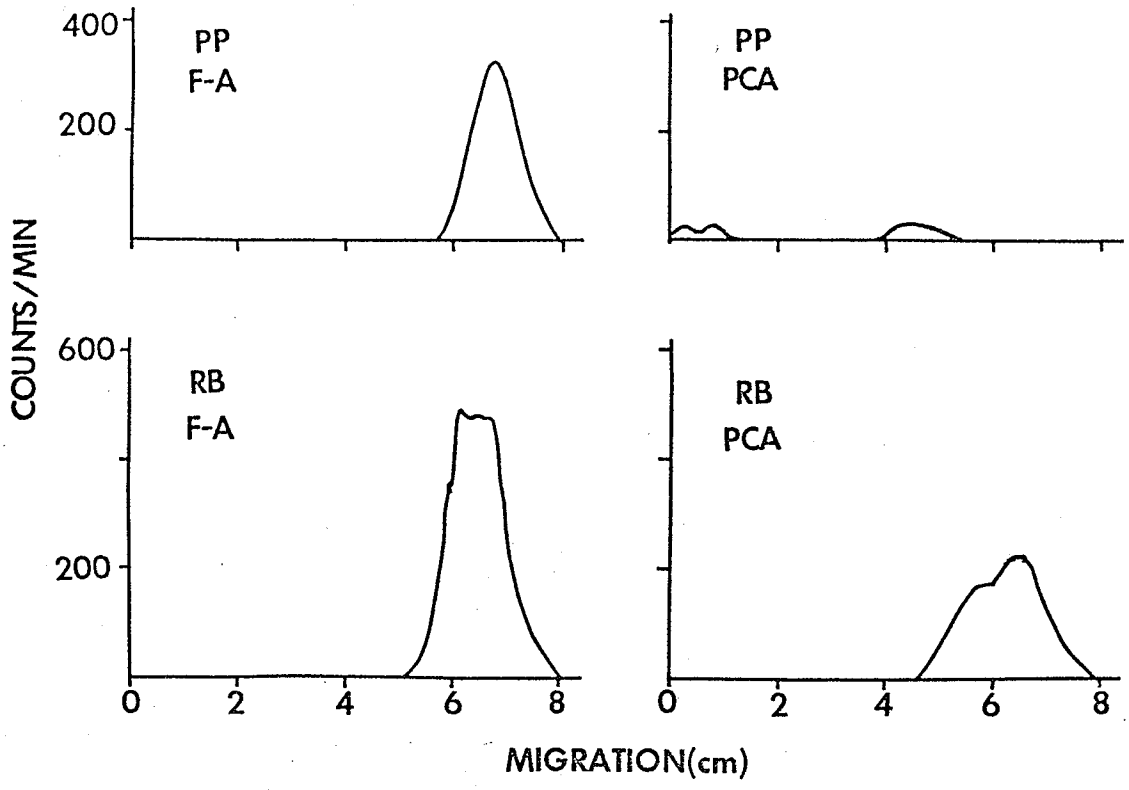

CPM
100
50
^{14}C-ACh + ^{14}C-Ch
+ 25µl RB extract
0
5
10
15
20
MIGRATION(cm)

Figure 3

Paper electrophoresis of ^{14}C-ACh added to PP and RB extracts. Formic acid-acetone (F-A) and perchloric acid (PCA) extracts were made of bovine posterior pituitary (PP) and rat brain (RB). Each extract was chromatographed on Dowex 1 anion-exchange resin and treated with warm ethanol prior to electrophoresis. Each paper strip that was scanned for radioactivity carried 1.25×10^{-3} μC_i of ^{14}C-ACh added to PP extract (corresponding to 25 mg fresh tissue).


400
200
PP
F-A
PP
PCA
COUNTS/MIN
600
200
RB
F-A
RB
PCA
0
2
4
6
8
0
2
4
6
8
MIGRATION(cm)

pituitary. Each of these extracts was treated with warm ethanol prior to paper electrophoresis, but the ethanol treatment was relatively unsuccessful in improving the characteristics of the perchloric acid extracts. The ^{14}C-ACh in the perchloric acid extract of rat brain shows poorer resolution than the ^{14}C-ACh in either of the formic acid-acetone extracts. The perchloric acid extract of bovine posterior pituitary had entirely unacceptable characteristics; some material remained at the origin, and most of the remainder was distributed so diffusely that a significant peak of ^{14}C-ACh radioactivity was not shown by the paper scanning.

A further experiment was done to see whether the electrophoresis characteristics of perchloric acid extracts of bovine posterior pituitary could be improved. Toru and Aprison (1966) had succeeded in improving the recovery of added ACh from another type of extract (acetic acid-ethanol extract of rat brain) by a double extraction technique. They treated the acetic acid-ethanol extract with formic acid-acetone. A similar double extraction procedure was applied here to perchloric acid extract of bovine posterior pituitary. Two types of double extraction were done: a lyophilized perchloric acid extract residue was treated with formic-acid acetone (P/F extraction), and a lyophilized formic acid-acetone extract residue was treated with perchloric acid (F/P

extraction). In both procedures the usual anion-exchange chromatography was done after the second extraction step. The lower half of Fig. 4 shows the electrophoretic migration patterns obtained with each of these double extracts before warm ethanol treatment. Both types of double extract gave unacceptable results without the ethanol treatment. A considerable amount of material remained at the origin, and the remainder was very diffusely distributed.

The upper half of Fig. 4 shows the electrophoretic migration patterns obtained with these double extracts after warm ethanol treatment and rejection of the resulting precipitates. Significant improvement was found with the P/F extract (although some material still remained at the origin), but there was little improvement to be seen with the F/P extract. The results shown in Figs. 3 and 4 indicate that where perchloric acid extraction was the last extraction step to be carried out before anion-exchange chromatography and ethanol treatment, satisfactory resolution of ^{14}C-ACh added to bovine posterior pituitary extracts was not obtained. The perchloric acid extraction technique was eliminated from further consideration in connection with the paper electrophoresis isolation of ACh in this study.

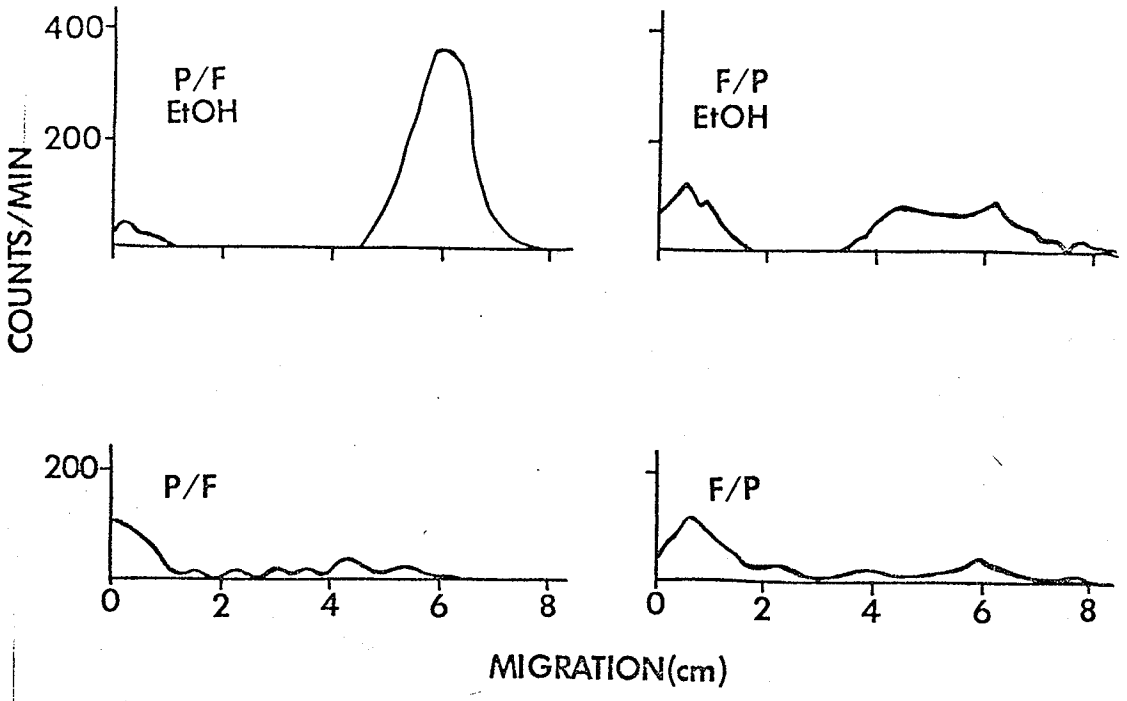
Stavinoha and Ryan (1965) had found that acetic acid-ethanol extracts of rat brain gave satisfactory

Figure 4

Paper electrophoresis of ^{14}C-ACh added to PP extracts. Double extractions of bovine posterior pituitary (PP). P/F indicates that a perchloric acid (PCA) extract residue was extracted with formic acid-acetone (F/A). F/P indicates that a F-A extract residue was extracted with PCA. Each paper strip scanned for radioactivity carried 1.25 X 10^{-3} μC_i of ^{14}C-ACh added to PP extract (corresponding to 160 mg fresh tissue).

Lower half of figure: First electrophoresis run of the extracts. Extracts had been treated with Dowex 1 anion-exchange chromatography resin, but no ethanol (EtOH) treatment had been done.

Upper half of figure: Second electrophoresis run of same extracts. Extracts had been treated with EtOH.


400
200
P/F
EtOH
F/P
EtOH
COUNTS/MIN
200
P/F
F/P
0
2
4
6
8
0
2
4
6
8
MIGRATION(cm)

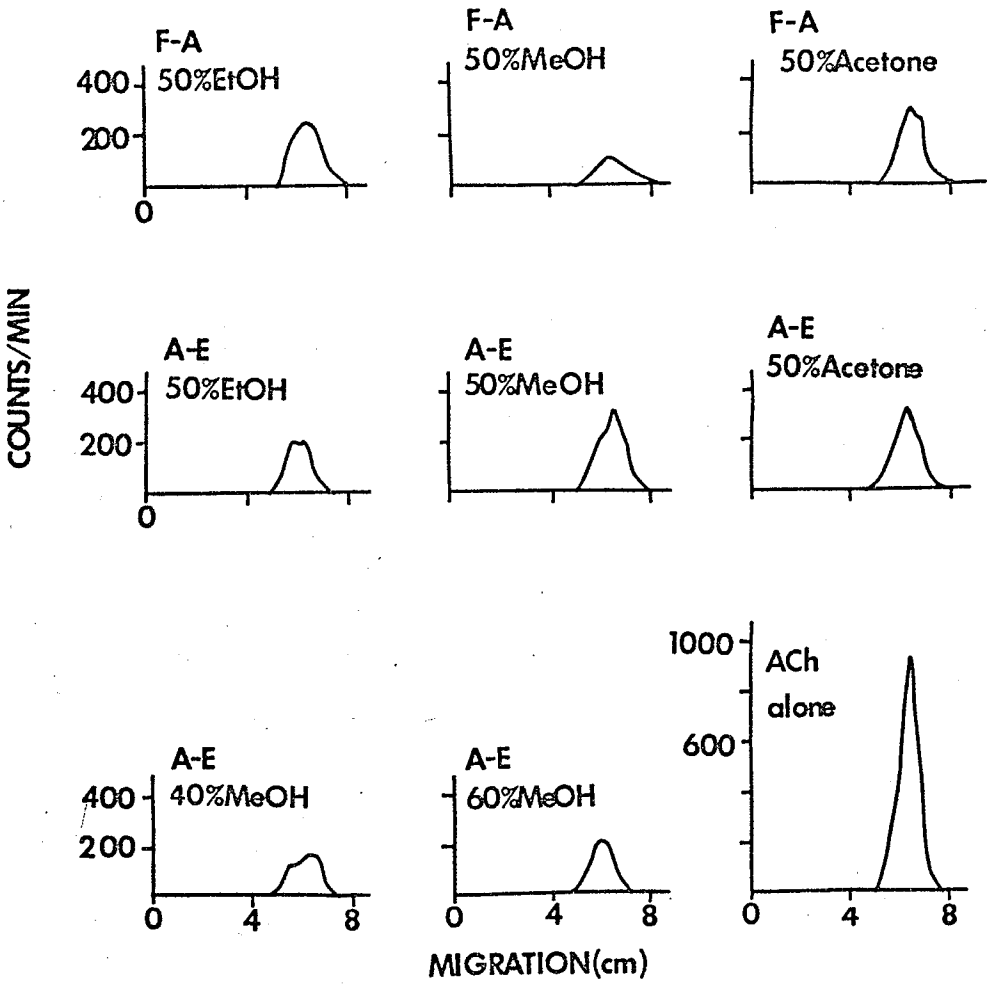
results with paper electrophoresis. No comparison had been made between acetic acid-ethanol extracts and formic acid-acetone extracts regarding suitability for paper electrophoresis. In the present study a comparison was made of the electrophoretic characteristics of bovine posterior pituitary extracts made by these two procedures.

In the course of experiments to compare the formic acid-acetone and the acetic acid-ethanol extracts, various organic solvent/water mixtures were tried as the solvent systems for the anion-exchange chromatography of the extracts. This was done because preliminary experments had shown that some such solvent mixtures were considerably more successful than others for use with the anion-exchange chromatographic removal of material that would have interfered with the electrophoretic mobility of ACh.

Fig. 5 shows the results of experiments comparing the electrophoresis characteristics of ^{14}C-ACh added to formic acid-acetone extracts and acetic acid-ethanol extracts of bovine posterior pituitary. The various solvent mixtures used in the anion-exchange chromatography procedure are indicated. Satisfactory electrophoresis characteristics were obtained with either formic acid-acetone extracts or acetic acid-ethanol extracts if the appropriate solvent system was used for anion-exchange chromatography prior to the electrophoresis. In order to avoid

Figure 5

Paper electrophoresis of ^{14}C-ACh added to PP extracts. The solvents (methanol, MeOH; ethanol, EtOH) were mixed in the indicated proportions (v/v) with water and used for Dowex 1 anion-exchange chromatography of extract prior to electrophoresis. Formic acid-acetone (F-A) or acetic acid-ethanol (A-E) extracts of bovine posterior pituitary (PP) were used. The paper strips scanned for radioactivity carried 1.25 X 10^{-3} μC_i of ^{14}C-ACh added to PP extract (corresponding to 50 mg fresh tissue) or 2 X 10^{-3} μC_i of ^{14}C-ACh alone.


F-A
50%EtOH
F-A
50%MeOH
F-A
50%Acetone
A-E
50%EtOH
A-E
50%MeOH
A-E
50%Acetone
A-E
40%MeOH
A-E
60%MeOH
ACh
alone
400
200
0
1000
600
4
8
COUNTS/MIN
MIGRATION(cm)

duplication of routine procedures, one of these two acceptable extraction procedures had to be chosen for routine use. The acetic acid-ethanol extraction procedure was adopted for routine use with paper electrophoretic isolation of ACh from tissue extracts.

Further experiments were done to study the electrophoresis characteristics of ^{14}C-ACh added to acetic acid-ethanol extracts of bovine posterior pituitary where methanol/water mixtures of various proportions were used as the solvent systems for the anion-exchange chromatography. There was some improvement of the electrophoresis characteristics with increasing proportions of methanol in these mixtures. Methanol concentrations higher than 70% (v/v) gave no further improvement of the effectiveness of anion-exchange chromatography. Seventy per cent methanol was adopted routinely for use as the solvent system with anion-exchange chromatography of acetic acid-ethanol tissue extracts prior to paper electrophoresis.

Fig. 6 shows the correspondence between the electrophoretic mobility of ^{14}C-ACh not added to tissue extract and the electrophoretic mobility of ^{14}C-ACh added to an acetic acid-ethanol extract of bovine posterior pituitary. Fig. 7 shows the electrophoretic resolution of ^{14}C-ACh added to acetic acid-ethanol extracts of bovine posterior pituitary, bovine anterior pituitary, bovine

Figure 6

Paper electrophoresis of ^{14}C-ACh alone or added to PP extract. The formic acid-acetone extract of bovine posterior pituitary (PP) was chromatographed on Dowex 1 anion-exchange resin prior to electrophoresis. Each paper strip scanned for radioactivity carried ^{14}C-ACh (2×10^{-3} μC_i) alone or ^{14}C-ACh (1.25×10^{-3} μC_i) added to PP extract (corresponding to 25 mg fresh tissue).

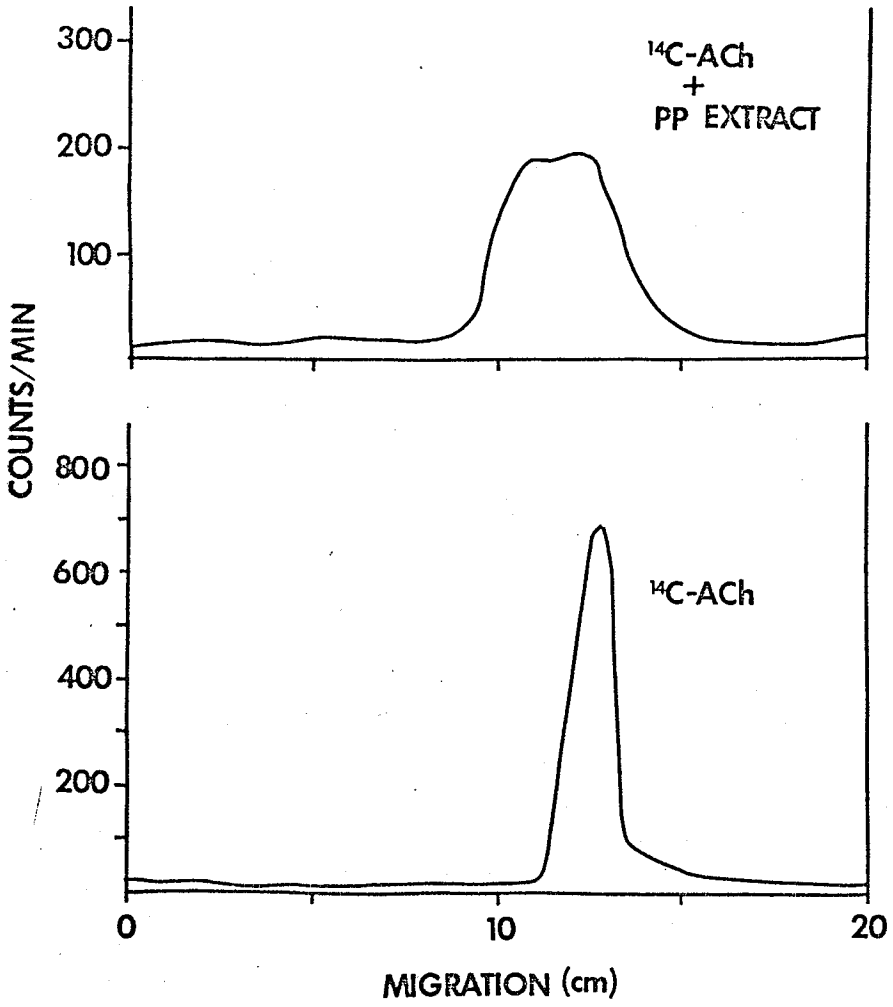
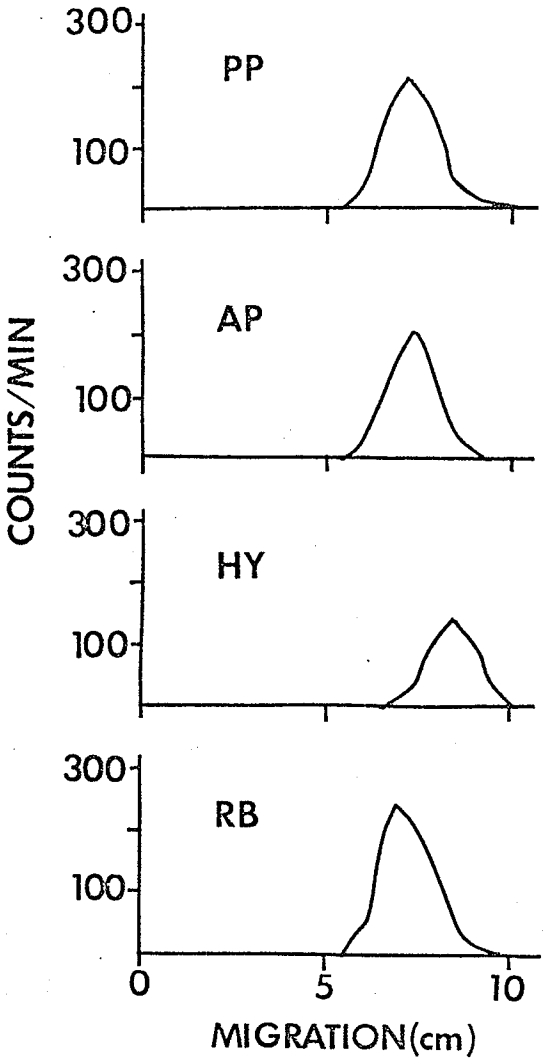

300
200
100
^{14}C-ACh
+
PP EXTRACT
COUNTS/MIN
800
600
400
200
^{14}C-ACh
0
10
20
MIGRATION (cm)

Figure 7

Paper electrophoresis of ^{14}C-ACh added to PP, AP, HY, and RB extracts. Formic acid-acetone extracts were made of rat brain (RB) and bovine posterior pituitary (PP), anterior pituitary (AP), and hypothalamus (HY). Each paper strip scanned for radioactivity carried 1.25×10^{-3} μC_i of ^{14}C-ACh. Each extract was chromatographed on Dowex 1 anion-exchange resin prior to electrophoresis.


300
100
PP
300
100
AP
300
100
HY
300
100
RB
COUNTS/MIN
0
5
10
MIGRATION(cm)

hypothalamus, and rat brain. The correspondence between the electrophoretic mobility of ^{14}C-ACh alone and that of ^{14}C-ACh added to tissue extract was good in the case of these extracts of bovine posterior pituitary, bovine anterior pituitary, bovine hypothalamus, and rat brain; this correspondence was similar to that shown in Fig. 6 for ^{14}C-ACh alone and ^{14}C-ACh added to bovine posterior pituitary extract.

The procedures used in the experiments from which results are shown in Figs. 6 and 7 were the routine procedures described in the Materials and Methods section. The electrophoretic patterns shown in Figs. 6 and 7 are representative of those found in all the experiments where paper electrophoresis was routinely used for the isolation of ACh from tissue extracts prior to isolated guinea-pig ileum bioassay.

CATION-EXCHANGE CHROMATOGRAPHY

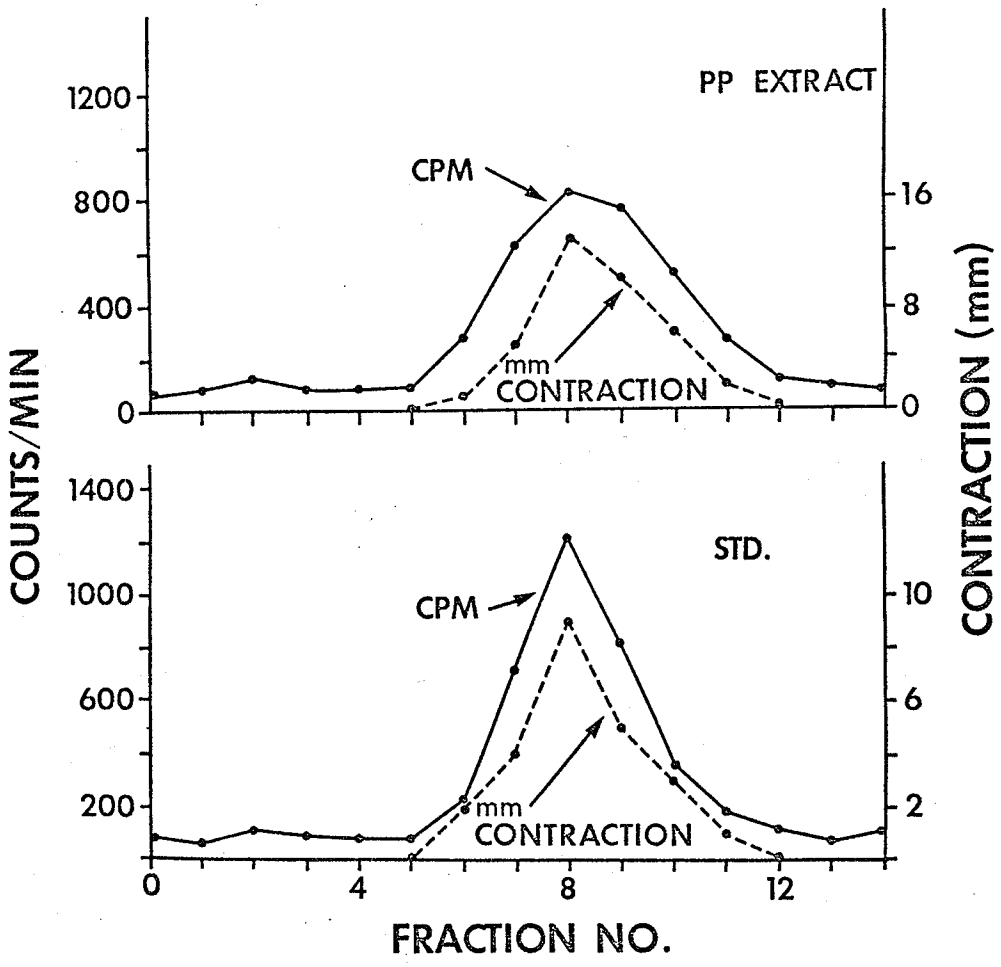
The cation-exchange chromatographic resolution of ^{14}C-ACh alone was found to be practically identical to that of unlabeled ACh alone (lower half of Fig. 8). The agreement was good between the cation-exchange chromatography characteristics of standard ACh alone (lower half of Fig. 8), ^{14}C-ACh added to a formic acid-acetone extract of bovine posterior pituitary (upper half of Fig. 8), and endogenous ACh found in a formic acid-acetone extract of bovine posterior pituitary (upper half of Fig. 8). Simi-

Figure 8

Cation-exchange chromatography of PP extracts and ACh. Two-ml fractions; 0.1 M NaH_2PO_4 was the eluting solvent. Dashed lines connect points indicating response of isolated guinea-pig ileum to 0.15-ml aliquots from the fractions. Solid lines connect points indicating radioactivity detected in 0.20-ml aliquots from the fractions.

Lower half of figure: Chromatography of 1.0 µg of unlabeled ACh alone (dashed line) and 10^{-2} μC_i of ^{14}C-ACh alone (solid line).

Upper half of figure: Chromatography of formic acid-acetone extract of bovine posterior pituitary (PP) alone (dashed line) and chromatography of PP extract with added ^{14}C-ACh (solid line). PP extract corresponding to 1.0 g fresh tissue was taken for each chromatography run (with or without 5×10^{-2} μC_i of added ^{14}C-ACh). Each PP extract was chromatographed on Dowex 1 anion-exchange resin prior to Bio-Rex 70 cation-exchange chromatography.


PP EXTRACT
CPM
mm
CONTRACTION
STD.
CPM
mm
CONTRACTION
COUNTS/MIN
CONTRACTION (mm)
1200
800
400
0
16
8
0
1400
1000
600
200
10
6
2
0
4
8
12
FRACTION NO.

larly good agreement was found between the cation-exchange chromatography characteristics of standard ACh alone and those of ^{14}C-ACh added to acetic acid-ethanol tissue extracts. Cation-exchange chromatography of perchloric acid tissue extracts was considerably less satisfactory than was the chromatography of either formic acid-acetone extracts or acetic acid-ethanol extracts.

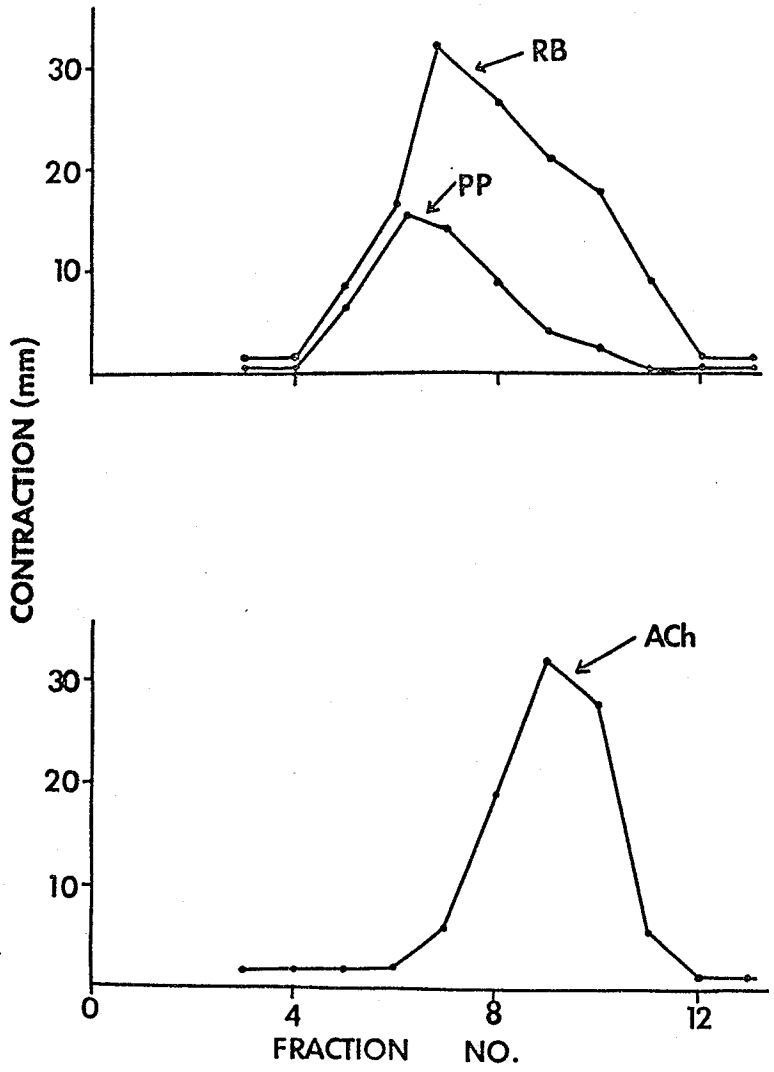
Fig. 9 shows the cation-exchange chromatographic resolution of ACh in perchloric acid tissue extracts compared with that of standard ACh alone. The results shown in Fig. 9 were obtained by the isolated guinea-pig ileum bioassay of individual cation-exchange chromatography fractions. The results from chromatography of unlabeled ACh alone are shown in the lower half of Fig. 9. The results from chromatography of perchloric acid extracts of bovine posterior pituitary and rat brain (upper half of Fig. 9) represent the endogenous tissue ACh found in the chromatography fractions. The results from chromatography of perchloric acid tissue extracts (Fig. 9) are to be compared with the results from chromatography of formic acid-acetone extracts (Fig. 8). The chromatography of formic acid-acetone extracts gave resolution of ACh (endogenous ACh or added ^{14}C-ACh) that was nearly identical to that of standard ACh alone (Fig. 8). Chromatography of the perchloric acid extracts gave resolution of ACh that was inferior to that of standard ACh alone

Figure 9

Cation-exchange chromatography of PP extract, RB extract, and standard ACh. Two-ml fractions; 0.1 M NaH_2PO_4 was the eluting solvent. Response of isolated guinea-pig ileum (mm contraction) to 0.15-ml aliquots from the fractions is shown.

Lower half of figure: Chromatography of 1.0 µg of standard unlabeled ACh alone.

Upper half of figure: Chromatography of perchloric extracts of bovine posterior pituitary (PP) and rat brain (RB). PP extract corresponding to 2.0 g of fresh tissue and RB extract corresponding to 1.4 g fresh tissue were taken for chromatography. Each extract was chromatographed on Dowex 1 anion-exchange resin prior to Bio-Rex 70 cation-exchange chromatography.


RB
PP
ACh
30
20
10
30
20
10
0
4
8
12
CONTRACTION (mm)
FRACTION NO.

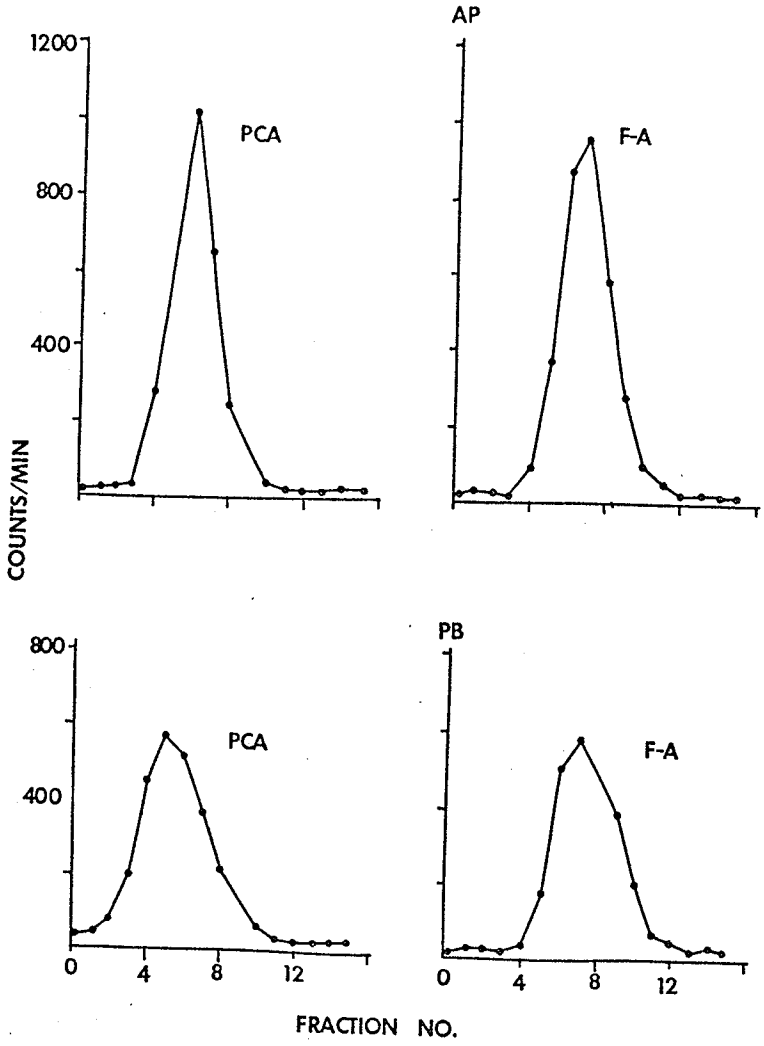
(Fig. 9). The ACh of perchloric acid extracts was distributed more diffusely through the chromatography fractions, and the peak of ACh activity was displaced to earlier fractions to a considerable extent with perchloric acid tissue extracts (Fig. 9).

Perchloric acid extracts of bovine anterior pituitary and pineal body also showed cation-exchange chromatography characteristics that were less satisfactory than the characteristics of formic acid-acetone extracts of these same tissues. The ^{14}C-ACh added to perchloric acid extracts of these tissues came off the columns more diffusely and with a peak in earlier fractions than the ^{14}C-ACh added to the formic acid-acetone extracts (Fig. 10).

In some experiments the ACh of perchloric acid tissue extracts came off the cation-exchange columns in such early fractions that there was, in some of these fractions, some biologically active material that interfered with the isolated guinea-pig ileum bioassay for ACh. The guinea-pig ileum stimulating activity of this interfering material was not abolished by 2.9×10^{-8} M atropine. This biologically active interfering material was found in the void volume of the columns; that is, it was not significantly retarded by the cation-exchange resin. In experiments where the ACh of perchloric acid tissue extracts began to come off the cation-exchange columns at a retention volume close to the void volume,

Figure 10

Cation-exchange chromatography of AP and PB extracts with added ^{14}C-ACh. Extracts of bovine anterior pituitary (AP) and pineal body (PB) were made with perchloric acid (PCA) or formic acid-acetone (F-A). Two-ml fractions collected; 0.1 M NaH_2PO_4 was the eluting solvent. Radioactivity detected in 0.20-ml aliquots of the fractions. Tissue extract corresponding to 1.0 g of fresh tissue with 5×10^{-2} μC_i of added ^{14}C-ACh was taken for each chromatography run. Each extract was chromatographed on Dowex 1 anion-exchange resin prior to the Bio-Rex 70 cation-exchange chromatography.


AP
1200
800
400
PCA
F-A
COUNTS/MIN
PB
800
400
PCA
F-A
0
4
8
12
0
4
8
12
FRACTION NO.

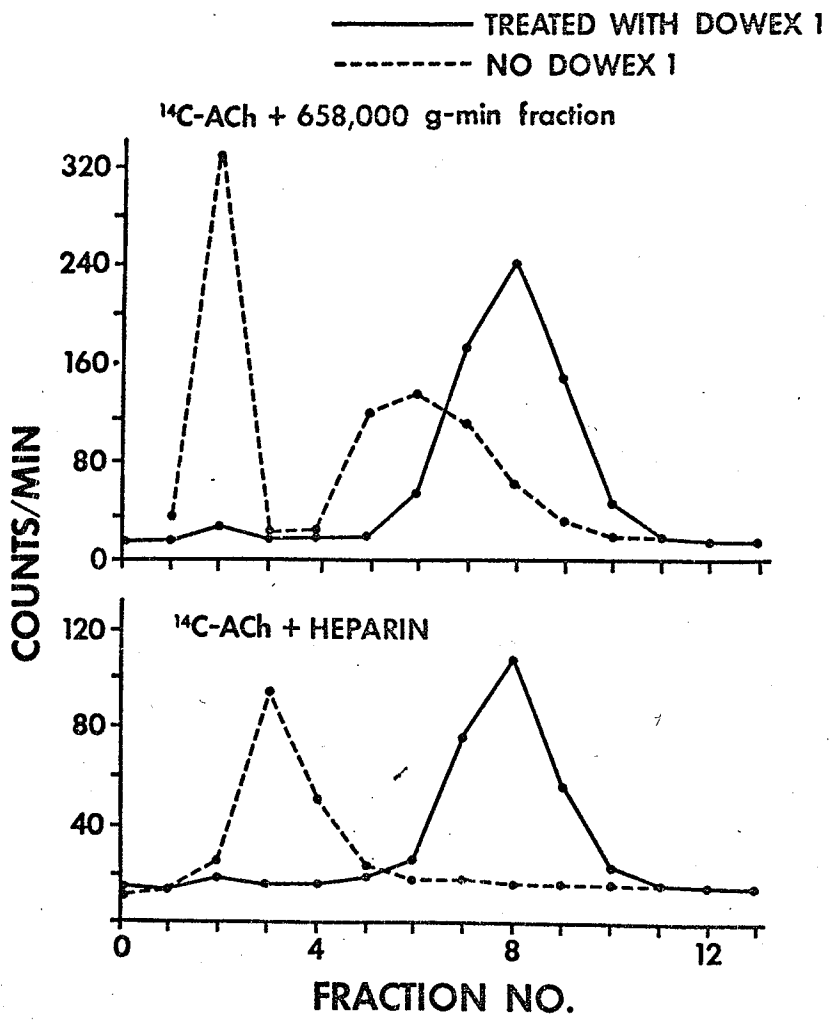
such early fractions were rejected from ACh bioassay and from estimation of ^{14}C-ACh recovery. Rejection of these early fractions from chromatography of perchloric acid tissue extracts allowed bioassay of the remaining ACh-containing fractions without contamination by biologically active interfering material.

The inferior cation-exchange chromatography characteristics of perchloric acid tissue extracts in comparison with extracts made with organic solvent systems (formic acid-acetone or acetic acid-ethanol) may likely be attributed to the presence of larger amounts of inorganic salts in the perchloric acid extracts. Whittaker (1963) has shown that the retention volume of ACh is displaced to earlier fractions by the presence of extraneous inorganic salt during cation-exchange chromatography.

The presence of a polyvalent anion can cause severe interference with the cation-exchange chromatography characteristics of ACh. The anion-exchange chromatography procedure used routinely in the present study eliminates such interference. Fig. 11 shows that Dowex 1 anion-exchange chromatography was effective in eliminating the severe interference that heparin can cause with the cation-exchange chromatography of ACh. The lower half of Fig. 11 shows the results obtained by Dowex 1 treatment of a mixture of ^{14}C-ACh and heparin without the presence

Figure 11

Cation-exchange chromatography of ^{14}C-ACh with heparin. Two-ml fractions; 0.1 M NaH_2PO_4 was the eluting solvent. Radioactivity was detected in 0.20-ml aliquots from the fractions. Solid lines indicate Dowex 1 anion-exchange treatment prior to Bio-Rex 70 cation-exchange chromatography. Dashed lines indicate that no Dowex 1 treatment was used. The solvent for Dowex 1 chromatography was 50/50 (v/v) acetone/water. Lower half of figure: Chromatography of 10^{-2} μC_i of ^{14}C-ACh in 50 ml of 0.29 M sucrose solution containing 0.02% (w/v) heparin.
Upper half of figure: Chromatography of 10^{-2} μC_i ^{14}C-ACh added to a formic acid-acetone extract of the 658,000 g-min fraction from differential centrifugation of 6.0 g bovine posterior pituitary in 0.29 M sucrose containing 0.02% (w/v) heparin.


TREATED WITH DOWEX 1
NO DOWEX 1
^{14}C-ACh + 658,000 g-min fraction
320
240
160
80
0
COUNTS/MIN
^{14}C-ACh + HEPARIN
120
80
40
0
4
8
12
FRACTION NO.

of tissue extract. The results shown in the upper half of Fig. 11 were obtained with cation-exchange chromatography of ^{14}C-ACh that had been added to a subcellular fraction from a bovine posterior pituitary homogenate. In this laboratory the procedures for centrifugation of tissue homogenates routinely include the addition of heparin to improve separation of subcellular particles (LaBella and Sanwal, 1965; Bindler, LaBella, and Sanwal, 1967). The upper half of Fig. 11 shows that without Dowex 1 anion-exchange treatment the cation-exchange chromatography of ^{14}C-ACh is quite unsatisfactory where the ^{14}C-ACh was added to a centrifugation fraction containing added heparin. The upper half of Fig. 11 also shows that the Dowex 1 anion-exchange treatment eliminates such interference. After elimination of the interference by heparin (upper and lower halves of Fig. 11) the resolution of ^{14}C-ACh is practically identical with that of ^{14}C-ACh chromatographed alone (Fig. 8).

The choice of the solvent system is important to the success of the anion-exchange chromatography of ACh in the presence of tissue extract and a polyvalent anion such as heparin. Preliminary experiments with various solvents showed that 50/50 acetone/water (v/v) gave optimal results with anion-exchange chromatographic removal of contaminating material from tissue extracts prior to the cation-exchange chromatography.

IDENTIFICATION OF ACh IN PURIFIED TISSUE EXTRACTS

Both the paper electrophoresis and the cation-exchange chromatographyisolation procedures for ACh gave purified tissue extracts free of material interfering with the guinea-pig ileum bioassay. The chemically purified tissue extracts stimulated the isolated guinea-pig ileum to contract, and such stimulation was abolished by a low concentration of atropine (2.9×10^{-8} M) or by incubation of the purified extract with AChE. Both of these tests of the identity of the active material (atropine antagonism and AChE inactivation) were applied routinely to each of the extracts of bovine posterior pituitary, anterior pituitary, and pineal body. They were applied also to the rat brain extracts.

In the single experiment with bovine hypothalamus, insufficient material remained after establishing the quantitative precision of the estimate to perform the usual tests of the identity of the active material. Thus, in the case of the bovine hypothalamus extract, the identity of ACh is assigned only tentatively to the biologically active material on the basis of its electrophoretic mobility (identical to ACh). Earlier work, however, has indicated that the ACh-like material isolated from bovine brain extracts by paper electrophoresis is attributable to ACh itself (see Discussion).

ESTIMATION OF ACh IN PURIFIED TISSUE EXTRACTS

Estimations of the recovery of ^{14}C-ACh added to the tissue extracts are given in Table 2. The ^{14}C-ACh was added at the time of tissue homogenization and carried through the routine extraction and isolation procedures. The recoveries ranged from 80% to almost 100%. Control experiments with unlabeled ACh added to homogenates of bovine posterior pituitary and anterior pituitary indicated similar values (75-95%) for the recovery of biologically active unlabeled ACh carried through the extraction and isolation procedures and assayed on the isolated guinea-pig ileum. Thus there was no indication of interference with estimates of endogenous ACh concentration in the tissue extracts by large losses of ACh during the purification procedure or by the presence of other material that would interfere with the bioassay procedure.

The estimates of endogenous ACh concentrations in extracts of bovine posterior pituitary, bovine anterior pituitary, bovine pineal body, bovine hypothalamus, and rat brain are given in Tables 3-7. In each case, except that of the central zone of the bovine anterior pituitary (Table 5), the extracts were made from the whole tissue indicated. The estimates of ACh concentration from all the tissues studied here are summarized in Table 8.

TABLE 2

RECOVERY OF ^{14}C-ACh ADDED TO TISSUE EXTRACTS

Extract	Purification	Per cent recovery				
		PP	AP	PB	HY	RB
Acetic acid-ethanol	P-E	(8) 92.2 ± 5.3	(3) 98.8 ± 3.8			(5) 90.7 ± 0.8
	C-E		(2) 91.6 ± 14.0*	(2) 82.8 ± 0.4	(1) 80.0	
Formic acid-acetone	C-E	(6) 96.2 ± 2.7	(4) 95.4 ± 6.2*	(2) 84.4 ± 2.0		
Perchloric acid	C-E		(2) 75.3 ± 3.3*	(2) 74.8 ± 0.4		

PP = bovine posterior pituitary; AP = bovine anterior pituitary; PB = bovine pineal body; HY = bovine hypothalamus; RB = rat brain (cerebrum); P-E = paper electrophoresis; C-E = cation-exchange.

Mean ± S.E. (No. of determinations) are shown.

* Central zone only of the AP was used.

TABLE 3

ACh IN ACETIC ACID-ETHANOL EXTRACTS OF PP PURIFIED BY PAPER ELECTROPHORESIS

Extract	Purification aliquot*	Assay sample	Tissue equiv. (mg)	Ileum preparation	Day of bioassay	[ACh] (nmoles/g fresh tissue) Assay sample	[ACh] (nmoles/g fresh tissue) Extract (mean)
1	1 + 2 + 3 + 4	a	90	1	1	2.6	2.5
	5 + 6 + 7 + 8	a	90	1	1	2.3	
2	1 + 2	a	125	1	2	1.4	2.4
	3 + 4	a	75	2	2	2.3	
	5 + 6	a	50	2	3	2.5	
	5 + 6	b	100	2	3	2.0	
	7 + 8	a	75	3	3	2.0	
	7 + 8	b	100	3	3	4.2	
3	1 + 2	a	47	1	4	1.9	2.0
	1 + 2	b	94	1	4	1.5	
	1 + 2	c	94	2	5	2.6	
	1 + 2	d	94	2	5	2.1	
	3 + 4	a	94	2	4	1.9	
	3 + 4	b	94	1	5	2.1	
	3 + 4	c	94	1	5	2.2	
Mean ± S.E.							2.3 ± 0.2

PP = bovine posterior pituitary.

Inter-extract variance relative to subsample variance was not significant. $F(2,12)$ 1.72 NS.

*Numbers refer to electrophoresis papers from which PP extract was taken and pooled for bioassay.

TABLE 4

ACh IN A FORMIC ACID-ACETONE EXTRACT OF PP PURIFIED BY CATION-EXCHANGE CHROMATOGRAPHY

Purification aliquot*	Assay sample	Tissue equiv. (mg)	Day of bioassay	[ACh] (nmoles/g fresh tissue) Assay sample	[ACh] (nmoles/g fresh tissue) Purification aliquot (mean)
1	a	125	1	1.0	
	b	50	2	1.2	1.1
	c	50	2	1.1	
2	a	150	1	0.9	
	b	150	1	0.7	0.8
3	a	150	1	1.8	
	b	150	1	1.4	1.5
	c	50	1	1.3	
4	a	50	1	0.6	
	b	100	1	0.5	1.2
	c	150	2	2.5	
Mean ± S.E.					1.2 ± 0.1

PP = bovine posterior pituitary.

Inter-extract variance relative to subsample variance was not significant. F(3,7) 1.89 NS.

*The numbers refer to cation-exchange columns from which PP extract ACh was taken for bioassay.

TABLE 5

ACh IN AP EXTRACTS

Tissue; extraction; purification	Extract	Purification aliquot#	Assay sample	Tissue equiv. (mg)	Ileum preparation	Day of bioassay	[ACh] (nmoles/g fresh tissue) Assay sample	Extract (mean)	Tissue (mean ± S.E.)
AP-whole gland A-E P-E	1	1 + 2 + 3 + 4	a	163	1	1	0.82	0.72	
		1 + 2 + 3 + 4	b	163	1	1	0.61		
	2	1 + 2	a	98	1	2	0.74		0.71 ± 0.01
		3 + 4	a	98	2	3	0.61		
		3 + 4	b	98	2	3	0.55		
		3 + 4	c	98	3	3	0.42	0.70	
		3 + 4	d	98	3	3	0.68		
		5 + 6	a	98	2	4	0.77		
		5 + 6	b	98	2	2	0.83		
AP-central zone F-A C-E	1	1	a	500	1	5	0.15		
		2	b	450	2	5	0.15	0.16	0.16 ± 0.01
		3	c	500	2	5	0.17		

AP = bovine anterior pituitary; A-E = acetic acid-ethanol extraction; F-A = formic acid-acetone extraction; P-E = paper electrophoresis purification; C-E = cation-exchange chromatography purification.

For whole AP, inter-extract variance relative to subsample variance was not significant. F(1,7) 3.90 NS.

#For AP (whole gland) the numbers refer to electrophoresis papers from which tissue extract ACh was taken and pooled for bioassay. For AP (central zone) the numbers refer to C-E columns from which

TABLE 6

ACh IN EXTRACTS OF PB PURIFIED BY CATION-EXCHANGE CHROMATOGRAPHY

Extracting solvent	Experiment	Assay sample	Tissue equiv. (mg)	Ileum preparation	Day of bioassay	[ACh] (nmoles/g fresh tissue) Assay sample	Experiment (mean)	Extract (mean + S.E.)
Formic acid-acetone	1	a	518	1	1	0.71	0.71	
	2	a	405	1	2	0.76		0.85 ± 0.13
		b	243	2	3	0.87	0.98	
		c	202	2	3	1.30		
Acetic acid-ethanol	1	a	414	1	4	0.55	0.55	
	2	a	404	1	5	0.33		0.46 ± 0.09
		b	252	1	6	0.40	0.37	
		c	303	2	6	0.39		
Perchloric acid	1	a	582	1	7	0.50	0.50	
	2	a	608	1	8	0.21		0.[illegible].14
		b	304	2	9	0.22	0.22	

PB = bovine pineal body.

TABLE 7

ACh IN ACETIC ACID-ETHANOL EXTRACTS OF BRAIN PURIFIED BY PAPER ELECTROPHORESIS

Tissue	Extract	Purification aliquot#	Assay sample	Tissue equiv. (mg)	Ileum preparation	Day of bioassay	[ACh] (nmoles/g fresh tissue) Assay sample	Extract (mean)	Tissue (mean ± S.E.)
Rat brain	1	1	a	28	1	1	5.8		
		2 + 3	b	56	2	1	4.9	5.4	
	2	1 + 2	a	9	1	2	6.9		
			b	10	1	3	6.4		6.2 ± 0.8
			c	10	2	3	11.3		
			d	21	1	2	6.9		
			e	21	1	3	10.8		
			f	21	1	3	4.8	7.0	
			g	21	1	3	5.5		
			h	21	1	3	5.7		
			i	21	2	3	6.6		
			j	21	2	3	6.2		
			k	34	3	4	6.3		
Bovine hypo-thalamus	1	1 + 2 + 3	a	78	1	5	1.5		
			b	155	1	5	1.2	1.4	1.4 ± 0.1
			c	155	2	5	1.5		

#The numbers refer to electrophoresis papers from which brain extract ACh was taken and pooled for bioassay.

TABLE 8

SUMMARY OF THE ACh CONTENT IN PITUITARY, PINEAL, AND BRAIN

Extract	Purification	[ACh] (nmoles/g fresh tissue) PP	AP	AP-CZ	PB	HY	RB
Acetic acid-ethanol	P-E	(8) 2.3 ± 0.2	(4) 0.71 ± 0.01			(3) 1.4 ± 0.1*#	(2) 6.2 ± 0.8
	C-E				(4) 0.46 ± 0.09		
Formic acid-acetone	C-E	(4) 1.2 ± 0.1		(3) 0.16 ± 0.01	(4) 0.85 ± 0.13		
Perchloric acid	C-E	(2) 1.4 ± 0.04#			(3) 0.36 ± 0.14		(2) 5.6 ± 0.1#

PP = bovine posterior pituitary; AP = bovine anterior pituitary; AP-CZ = central zone of AP; PB = bovine pineal body; HY = bovine hypothalamus; RB = rat brain (cerebrum); P-E = paper electrophoresis; C-E = cation-exchange chromatography.

Mean ± S.E. (no. of determinations) are shown. The no. of determinations refers to the sub-samples brought to bioassay.

* Antagonism by atropine not tested.

Inactivation by AChE not tested.

IV. DISCUSSION

EXTRACTION AND ISOLATION OF ACh

Various extraction methods were compared for ACh in the tissues studied here for the first time. Both paper electrophoresis and cation-exchange chromatography were used for isolating ACh from tissue extracts. Although both methods have proved useful for eliminating material that interferes with the bioassay of ACh (Whittaker, 1963; Potter and Murphy, 1967), they have not been previously compared for a single tissue.

In the present study ACh was more clearly resolved from rat brain extract than from extracts of any other tissue, either by paper electrophoresis or cation-exchange chromatography. This finding suggested the necessity of careful purification of the bovine tissue extracts. The extraction and isolation procedures for ACh that were developed and evaluated in this study (see Results) provided effective purification of the ACh in tissue extracts.

IDENTIFICATION OF ACh

The guinea-pig ileum stimulating material in the extracts studied here showed paper electrophoretic characteristics and cation-exchange chromatography characteristics identical to those of ^{14}C-ACh added to the tissue extracts. Likewise, the material with guinea-pig ileum stimulating activity in these purified tissue extracts, like authentic ACh, was rapidly inactivated

by boiling at pH 10, but not at pH 4. Furthermore, the guinea-pig ileum stimulating activity of the purified tissue extracts was completely abolished by a concentration of atropine (2.9×10^{-8} M) known (Gaddum and Picarelli, 1957; Cheema, 1965) to block the guinea-pig ileum stimulating effect of ACh but not that of histamine. The usefulness of atropine antagonism in identification of active material in the guinea-pig ileum bioassay has been discussed by Whittaker (1963).

Inactivation studies with erythrocyte AChE on extracts of bovine posterior pituitary, bovine anterior pituitary, bovine pineal body, and rat brain indicated the active material to be ACh or a closely related ester. Erythrocyte AChE hydrolyzes butyrylcholine (BuCh) and higher homologues of ACh at a low rate or not at all (Augustinsson, 1963). Furthermore, propionylcholine (PrCh), BuCh, and other choline esters are relatively inactive on the isolated guinea-pig ileum (Whittaker, 1963). Thus the choline ester content of tissue extracts in this study is estimated in terms of ACh concentration, since it is unlikely that other choline esters were present. In the case of rat brain extracts, others (Stavinoha and Ryan, 1965; Toru and Aprison, 1966) have used AChE inactivation and atropine antagonism of guinea-pig ileum activity to confirm that the material with electrophoretic and chromatographic mobility identical

to ACh was indeed ACh.

The experiment with bovine hypothalamus extract allowed only tentative identification of the biologically active material as ACh. It showed paper electrophoretic mobility identical to that of ACh, but atropine antagonism and AChE inactivation were not studied. Henschler (1956), using a paper electrophoresis technique, and Keyl (1957), using cation-exchange chromatography, established that the only choline ester present in fresh bovine brain extracts was ACh.

ACh CONTENT OF BOVINE PITUITARY AND PINEAL BODY

This report presents the first detailed estimation of the ACh content of bovine posterior pituitary. Lederis and Livingston (1969) carried out a detailed study of ACh in rabbit posterior pituitary, and they made some observations also on the posterior pituitary of other species. They considered their extraction technique to be inadequate, however, for tissues from species other than the rabbit. Estimation of ACh content is reported here for the first time in the case of the anterior pituitary and the pineal body.

Estimates for ACh of the bovine posterior pituitary were higher in extracts purified by electrophoresis (Table 3) than in extracts purified by cation-exchange chromatography (Table 4). This difference may be due to the presence of sodium dihydrogen phosphate in

methanol extracts of lyophilized cation-exchange chromatography fractions. Control experiments showed that this salt depresses the response of the isolated guinea-pig ileum to ACh.

Isolation of ACh by paper electrophoresis of bovine posterior pituitary extracts was carried out over a period of a few months, and differences of sampling (tissues) might be expected to occur. Analysis of variance (Steel and Torrie, 1960), however, shows that the differences among the means of the three experiments using paper electrophoresis of bovine posterior pituitary extracts are not significant ($F(2,12)$ 1.72).

It does not seem likely that the difference in extraction procedures would account for the higher estimate of ACh content of bovine posterior pituitary with paper electrophoresis (acetic acid-ethanol extract) compared to cation-exchange chromatography purification (formic acid-acetone extract). Others (Toru and Aprison, 1966) have indicated that for rat brain the formic acid-acetone extraction in fact gives higher estimates of the ACh concentration of tissue extract than does acetic acid-ethanol extraction.

Analysis of the central zone of the bovine anterior pituitary was done after other experiments with whole bovine anterior pituitary had shown an appreciable concentration of ACh. In the bovine anterior pituitary,

extensive innervation is not demonstrable histologically (Gilmore et al., 1941; Ribas-Mujal, 1958). However, ACh in the anterior pituitary might be due to the presence of cholinergic autonomic nerves from the capsule of the gland. Approximately five times more ACh was found for the whole bovine anterior pituitary than for the central zone (Table 5). This likely reflects true differences in the ACh concentrations of these two regions of tissue rather than differences in sampling of bovine pituitaries or in extraction and purification techniques. Analysis of variance shows the difference between means of the experiments with whole bovine anterior pituitary to be not significant ($F(1,7)$ 3.90). The ACh estimate from paper electrophoresis purification (acetic acid-ethanol extract) was only twice that of cation-exchange chromatography purification (formic acid-acetone extract); Tables 3 and 4.

The ACh content for the central zone of bovine anterior pituitary is very low. Indeed, it is so low that involvement of the ACh metabolizing system of blood should be considered. Presumably the one hour's transportation time to the laboratory for the bovine tissues would have allowed for hydrolysis of ACh originally in the bovine blood, but conflicting reports exist concerning ACh synthesis in blood (Koelle, 1963). The sensitivity of the ACh bioassays has apparently not ruled out

conclusively either the presence of ACh (Chang and Gaddum, 1933) or ChAc (Koelle, 1963) in blood. Even if a significant fraction of the ACh in the bovine anterior pituitary central zone were due to synthesis in blood, the five-fold higher ACh content of whole anterior pituitary could not be attributed entirely to blood ACh. The higher concentration of ACh in the peripheral zone of the anterior pituitary might be related to cholinergic autonomic nerves from the capsule of the gland or to differences in the ACh metabolizing and storage characteristics of the central and peripheral zones of the anterior pituitary.

COMPARISON OF ACh ESTIMATES IN THE PRESENT STUDY WITH THOSE REPORTED BY OTHERS

Direct comparison of the present results with those of others is possible only for bovine posterior pituitary, bovine hypothalamus, and rat brain, since ACh content for anterior pituitary and pineal body has not been reported previously. The ACh content of pituitary, pineal, and brain as estimated in the present study is presented in Table 8.

The present estimates for the ACh content of bovine posterior pituitary are 1.2-2.3 nanomoles ACh/gram of fresh tissue, whereas Lederis and Livingston (1969) reported 1.0 nanomole ACh/gram of bovine posterior pituitary. The estimate for bovine hypothalamus from

the present study is 1.4 nanomoles ACh/gram of tissue. Lederis and Livingston (1969) reported 1.0 nanomole ACh/gram of bovine hypothalamus.

The present estimates for the ACh content of bovine posterior pituitary and hypothalamus agree well with the estimates of Lederis and Livingston (1969) for these tissues. Lederis and Livingston (1969) considered, however, that the extraction procedure which they used for these bovine tissues was inadequate. They used HCl-eserine extraction for bovine posterior pituitary and hypothalamus, but in their study of rabbit posterior pituitary and hypothalamus they reported that perchloric acid extracts gave ACh estimates some twenty-fold to forty-fold higher than did HCl-eserine.

The twenty-fold increase in ACh estimates reported by Lederis and Livingston (1969) with perchloric acid extraction seems quite remarkable. In the present experiments with perchloric acid extraction (bovine posterior pituitary, bovine pineal body, and rat brain), ACh levels were found to be no higher than for other types of extracts of these tissues (Table 8). Others (Morris et al., 1965; Bull, Morris, and Hebb, 1969) have reported either no increase or no more than three-fold to four-fold increases in ACh estimates with perchloric acid extracts as compared with other extracts. Bull et al., (1969) found that perchloric acid and trichloroacetic acid extracts gave nearly identical estimates

for the ACh content of the electric organ of Torpedo, while acetic acid-ethanol extraction gave values only 1/3 or 1/4 as high. Since trichloroacetic acid extraction and perchloric acid extraction give nearly identical ACh estimates (electric organ of Torpedo), indirect comparison of perchloric acid and HCl-eserine extract ACh concentrations may be arrived at by noting that, for rat brain, both the HCl-eserine extracts and the trichloroacetic acid extracts give similar ACh estimates (Table 9).

The use of different bioassay preparations is not likely to account for the fact that, while no higher values for ACh were found in perchloric acid tissue extracts in the present study, the estimates of Lederis and Livingston (1969) showed twenty-fold higher values for perchloric acid extraction. For bioassay Lederis and Livingston (1969) used the leech muscle preparation, whereas the isolated guinea-pig ileum was used in the present study. Bull et al., (1969) found, however, that both the leech muscle and the guinea-pig ileum give similar results for a given type of extract (perchloric acid or acetic acid-ethanol).

Although 100% inactivation by AChE has been found for the guinea-pig ileum stimulating activity of purified tissue extracts (Toru and Aprison, 1966; present study), Lederis and Livingston (1969) found only 79-82%

TABLE 9

LITERATURE VALUES FOR THE ACh CONTENT OF SEVERAL TISSUES

Tissue	Extraction	Purification	[ACh] nmoles/g fresh tissue	Species	Reference
Whole brain	F-A	P-C	29.6	Rat	Toru and Aprison (1966)
	A-E	P-C	20.8	Rat	Toru and Aprison (1966)
	A-E	P-E	13.9	Rat	Stavinoha and Ryan (1965)
	A-E	P-E	6.2	Rat	Present study
	TCA	None	15.1	Rat	Toru and Aprison (1966)
	H-E	None	15.0	Rat	Takahashi et al. (1961)
	PCA	C-E	5.6	Rat	Present study
	TCA	C-E	1.0	Ox	Keyl (1957)
	TCA	C-E	8.6	Rabbit	Keyl (1957)
Hypothalamus	A-E	None	20.3*	Rat	Aprison et al. (1968)
	A-E	P-E	1.4	Ox	Present study
	H-E	None	1.0	Ox	Lederis and Livingston (1969)
	H-E	None	0.8	Rabbit	Lederis and Livingston (1969)
	PCA	None	33.4	Rabbit	Lederis and Livingston (1969)

Posterior pituitary	F-A	C-E	1.2	Ox	Present study
	A-E	P-E	2.3	Ox	Present study
	PCA	C-E	1.4	Ox	Present study
	H-E	None	1.0	Ox	Lederis and Livingston (1969)
	H-E	None	1.4	Rabbit	Lederis and Livingston (1969)
	PCA	None	30.0	Rabbit	Lederis and Livingston (1969)
Electric organ	A-E	-	13.0	Electric eel	Bull et al. (1969)
	TCA	-	50.0	Electric eel	Bull et al. (1969)
	PCA	-	49.4	Electric eel	Bull et al. (1969)

F-A = formic acid acetone; A-E = acetic acid-ethanol; TCA = trichloracetic acid;
H-E = HCl-eserine; PCA = perchloric acid; P-C = paper chromatography;
P-E = paper electrophoresis; C-E = cation-exchange chromatography.

*This [ACh] estimate was for diencephalon plus mesencephalon.

inactivation of active material in perchloric acid extracts and 77-91% inactivation for HCl-eserine extracts. Incomplete inactivation by AChE of active material in their boiled HCl-eserine extracts may be related to the fact that eserinized and boiled tissue extracts are known (MacIntosh, 1939) to interfere with the leech muscle bioassay by sensitizing the preparation to ACh.

The perchlorate ion sensitizes the isolated frog rectus abdominis muscle to ACh (Hanin and Jenden, 1966). Although the leech muscle is considered to be less sensitive than the frog rectus abdominis muscle to interfering material (MacIntosh and Perry, 1950), there is some question about possible interference with the leech muscle bioassay by perchlorate. Certain salts are known (MacIntosh and Perry, 1950) to sensitize the leech muscle to ACh.

Sensitization of isolated muscle preparations to ACh by concentrations of interfering substances that do not themselves produce responses has been reported by MacIntosh and Perry (1950) and by Stockley (1969), but Lederis and Livingston (1969) did not indicate whether ACh added to their tissue extracts gave equal or greater leech muscle stimulation than the ACh alone. In the case of the guinea-pig ileum bioassay of purified tissue extracts (Stavinoha and Ryan, 1965; Toru and Aprison, 1966; present study), ACh added to purified tissue

extracts shows the same biological activity as ACh not added to the extract.

One point of agreement between the results of the present study and the results reported by Lederis and Livingston (1969) is that, with a given extraction procedure, the estimates of ACh content for posterior pituitary and hypothalamus of a given species are similar. There is agreement also between the ACh content of bovine posterior pituitary and bovine hypothalamus as found in the present study and the ACh content found previously for whole bovine brain (Keyl, 1957; Table 9). For the rat brain also the ACh content of whole brain is similar to that of the hypothalamus (Toru and Aprison, 1966; Aprison et al., 1968; Table 9). Neither of the two estimates given by Lederis and Livingston (1969) for ACh content of rabbit hypothalamus, however, is in agreement with the ACh content found for whole rabbit brain (Keyl, 1957; Table 9).

ACh estimates from different laboratories for purified extracts of rat brain are presented in Table 9. There was no indication in any of the present experiments with different extraction procedures (perchloric acid, acetic acid-ethanol, or formic acid-acetone) or purification procedures (paper electrophoresis and cation-exchange chromatography) of an ACh concentration greater than the value of 6.2×10^{-9} mole ACh/gram of fresh rat

brain, the estimate given in Table 9 from the results of the present study. Therefore, variations in extraction or purification procedures alone do not appear to account for the considerable difference between the present estimate and the estimates of others for rat brain (Table 9). The strain of rats used may conceivably contribute to these differences. The differences (Bennett et al., 1960) among inbred rat strains, however, seem relatively small (differences on the order of 20% of the total values). Thus it is not yet established whether strain differences (albino rats used by others; hooded rats used in the present study) could account for ACh estimate differences between laboratories on the order of 100% (Table 9).

SIGNIFICANCE OF ACh AND RELATED ENZYMES IN BOVINE PITUITARY AND PINEAL BODY

LaBella and Shin (1968) reported ChAc, AChE, and butyrylcholinesterase (BuChE) activity in bovine posterior pituitary, anterior pituitary, and pineal body. They related the activities of these enzymes in the bovine tissues to the activities of the same enzymes in rat brain. They found similar low levels of BuChE activity in each of the tissues studied when the tissues were washed to remove blood. ChAc and AChE activities were much higher in rat brain than in the bovine posterior pituitary, anterior pituitary, and pineal body. The activities in posterior pituitary were higher than in either pineal

body or anterior pituitary.

In the present study, rat brain was found to have a higher ACh concentration than bovine posterior pituitary or pineal body. The ACh concentration of anterior pituitary was the lowest of all the tissues studied. These results indicate general parallelism with the results of LaBella and Shin (1968). This parallelism is in line with the findings of others for nervous tissue (Koelle, 1969) and for non-innervated tissue such as the human placenta (Ord and Thompson, 1950; Keyl, 1957; Bull, Hebb, and Ratkovič, 1961).

Lederis and Livingston (1969) reported that the AChE activity of rabbit posterior pituitary was higher than that in bovine posterior pituitary (as reported by LaBella and Shin, 1968). This finding does not seem surprising in view of the fact that earlier work (Nachmansohn, 1940; Feldberg, 1945) showed that, for almost all brain regions where cholinesterase (ChE) activities of bovine and rabbit tissue were compared, the rabbit tissue had about two times the activity of the bovine tissue.

The BuChE levels found for rat brain, bovine posterior pituitary, bovine anterior pituitary, and bovine pineal body (washed to remove the BuChE of blood) as reported by LaBella and Shin (1968) are similar to the BuChE levels reported for rabbit posterior pituitary by

Lederis and Livingston (1969). This similarity of BuChE levels for various tissues suggests a common function for BuChE in these tissues. Since Lederis and Livingston (1969) did not indicate that they had washed the tissues they examined, the higher values they found for BuChE in rabbit hypothalamus may have reflected contaminating blood enzyme. Their rabbit posterior pituitary samples may possibly have been smaller than their rabbit hypothalamus samples, so that the BuChE activity of adhering blood would have been less significant.

The biochemical findings that bovine posterior pituitary has higher AChE activity than bovine anterior pituitary (LaBella and Shin, 1968) are in agreement with similar histochemical findings on the AChE activity of these tissues (Arvy, 1961b). Although it is not clear what means were used by Arvy (1961b) to evaluate the cholinesterase (ChE) specificity, she reported that the AChE activity of bovine pars distalis was weaker than the AChE activity of bovine pars nervosa.

ChE activity in bovine pars nervosa was reported by Arvy (1961b, 1964) to predominate in dorsally situated fibers. The possible contribution of activity in the pars intermedia to the levels of ACh, ChAc, and AChE in the posterior pituitary remains to be evaluated. It would seem from the findings of Arvy (1961b) that non-specific esterases may account for the hydrolysis of acetylthio-

choline (ASCh) in bovine pars intermedia.

Arvy (1961b) found that the ChE of the medullary region of the bovine anterior pituitary is more active toward butyrylthiocholine, while the paramedian zone is more active toward ASCh. This suggests functional significance for the different ACh concentrations indicated for peripheral and central regions of bovine anterior pituitary in the present study.

AChE activity in the bovine pineal body has been studied histochemically by Arvy (1961a, 1965), who concluded that the enzyme is primarily confined to fine nerve fibers accompanying larger blood vessels in the postero-ventral portion of the pineal body. Other AChE-containing nerve fibers that she described were scattered myelinated fibers passing into and back out of the pineal body in the habenular and caudal commissures described by Kappers (1965). Arvy (1965) also noted that the AChE-containing fibers in these commissures penetrate hardly at all into the pineal body parenchymal tissue.

POSSIBLE PHYSIOLOGICAL ROLES FOR THE ACh SYSTEM IN ENDOCRINE TISSUES

The levels of activity of the ACh system (ACh, ChAC, and AChE) show a parallelism with the extent of innervation for the bovine posterior pituitary, pineal body, and anterior pituitary: posterior pituitary greater than pineal body, and anterior pituitary less than either

of the other two tissues. The innervation of the pineal body and anterior pituitary is almost entirely autonomic, and thus the ACh system of these tissues is likely associated with their autonomic nerve supply. There are some observations to suggest that the ACh system in the posterior pituitary also is related to autonomic innervation.

In the posterior pituitary, the massive innervation by neurosecretory neurons of the hypothalamo-hypophysial tract is much greater in extent than the autonomic innervation (Dandy, 1913; Rasmussen, 1938; Gilmore et al., 1941; Christ, 1966). Although at least some of the neurosecretory neurons terminating in the posterior pituitary may be cholinoceptive (Duke et al., 1950; Daniel and Lederis, 1966a), there is now little or no reason to believe that they are cholinergic (Douglas and Poisner, 1964; Daniel and Lederis, 1966a; Dicker, 1966; Shute and Lewis, 1966b; Mikiten, 1967).

Differential centrifugation of bovine posterior pituitary homogenates has indicated that ACh is highest in isolated nerve endings and microvesicle fractions (LaBella, 1968). Thus, ACh is apparently localized in microvesicles analogous to "synaptic vesicles" of other nerve tissue (Whittaker, 1968), and these microvesicles are located within nerve terminals of bovine posterior pituitary. The possible implications of these biochemical

findings, together with appropriate ultrastructural findings, have been discussed by LaBella (1968), who believes that there may be separate populations of neurosecretory nerve endings and cholinergic non-neurosecretory nerve endings in the bovine posterior pituitary. Lederis (1967a) and Lederis and Livingston (1968) have interpreted their preliminary data on the subcellular distribution of ACh in rabbit posterior pituitary to suggest that at least some ACh is localized in non-neurosecretory nerve endings. The non-neurosecretory nerve endings containing ACh in the mammalian posterior pituitary may be autonomic nerve terminals serving a function of vascular regulation.

It is likely that ACh and related enzymes in bovine pineal body are associated primarily with the AChE-containing nerve fibers described by Arvy (1961a; 1965). Positive correlations have usually been found between the intensity of histochemical AChE activity of nervous tissue and the levels of AChE, ChAc, and ACh found in the same tissue by biochemical methods (Koelle, 1969). The AChE-containing nerve fibers accompanying larger blood vessels of the pineal (Arvy, 1965) are probably autonomic fibers. The AChE-containing fibers of the habenular and caudal commissures in the vicinity of the pineal body would, according to the interpretation of Kappers (1965), not be directly related to pineal body parenchymal cell function. Since the autonomic innervation of the pineal

body may have an important role in the physiology of that tissue (Kappers, 1965; Wurtman et al., 1968), it may be that the ACh system associated with autonomic nerve fibers to the pineal could have some significant physiological role.

The extent of innervation of the mammalian anterior pituitary is small. There is no secretomotor nerve supply to the mammalian anterior pituitary (Harris, 1955; Green, 1966a), and the few nerves in the anterior pituitary appear to be autonomic nerves related to vascular control (Rasmussen, 1938; Szentágothai et al., 1962). The ACh system in the mammalian anterior pituitary is probably associated with such autonomic nerve fibers. The marked species differences in levels and apparent specificity of ChE's of mammalian anterior pituitary (Arvy, 1963) suggest that there may be considerable variation from one species to another in levels of activity of the ACh system in the mammalian anterior pituitary.

The avian anterior pituitary and the mammalian anterior pituitary may be very different in regard to the importance of the ACh system for physiological function. A striking correlation between AChE activity and endocrinological activity has been demonstrated in the anterior pituitary of birds (Russell and Farner, 1968; Russell, 1968; Haase and Farner, 1969), but no striking changes of ACh system activities have been correlated

with anterior pituitary function in mammals. Dumont (1956) found that certain cells of the rabbit anterior pituitary showed histochemical AChE activity, but this activity was not clearly correlated with endocrinological activity. Sawyer and Everett (1947) found differences in AChE activity between the female and the male in the case of the rat anterior pituitary, but Kobayashi et al., (1964) did not find notable differences from one stage of the estrous cycle to another in the ChAc activity of pooled whole pituitaries from female rats.

Unfortunately, the studies of histochemically demonstrable AChE activity in mammalian pituitary have not generally been accompanied by corresponding determinations of ChAc and ACh. The study on bovine posterior pituitary, anterior pituitary, and pineal body (LaBella and Shin, 1968; present study) has provided biochemical data that may be related to histochemical findings of AChE activity in these tissues (Arvy, 1963).

More detailed information is necessary on correlations of endocrine function and ACh system activity before it will be possible to establish the involvement or lack of involvement of the ACh system in endocrine tissue function. Lederis (1967b) attempted to correlate the several hundred-fold variation in his ACh content estimates for rabbit posterior pituitaries with the physiological state of the posterior pituitary. He stimulated

release of neural lobe hormones with ether anesthesia and hemorrhage, but such experiments failed to show significant differences in ACh content of the posterior pituitary between stimulated animals and controls. Russell and Farner (1968), Russell (1968), and Haase and Farner (1969) have found correlations of AChE activity and anterior pituitary function in birds; thus it may be that estimation of ACh and ChAc activity of the avian pituitary would materially help to answer the questions about the possibility of an involvement of the ACh system in certain endocrine tissue functions.

The human placenta is a tissue with endocrinological function (Lloyd, 1968). Although the human placenta may be considered to be a tissue that does not receive innervation (Hebb, 1961), it contains high levels of ACh, ChAc, and AChE activity (Ord and Thompson, 1950; Keyl, 1957; Bull et al., 1961). Bull et al., (1961) found large variations in the ChAc activity of human placenta with the time course of pregnancy, and they suggested the possible involvement of ChAc in control of the transport of materials across the barrier separating maternal and fetal blood. As Koelle (1963) has pointed out, however, further experimental evidence is required before any broad generalization is possible about the question of involvement of the ACh system in control of permeability of biological membranes.

There are marked species differences in the levels of ACh, ChAc, and AChE for certain tissues (Chang and Gaddum, 1933; Koelle, 1963; Arvy, 1963; Hebb and Ratković, 1964; Russell, 1968; Welsch and Pearse, 1969). This suggests that the physiological significance of the ACh system in a given tissue may vary from one species to another.

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