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Cholinergic Systems in Non-nervous Tissues

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I. Introduction

Acetylcholine (ACh) seems to be the most ancient chemical transmitter and is a mediator in a wide variety of animals in which chemical transmission plays a significant role (293). The role of ACh as a chemical transmitter at a number of sites in the central and peripheral nervous systems of higher vertebrates has been well established. It has become popular to refer to the nerves that release ACh from their terminals as cholinergic nerves and to the nervous system in which ACh is involved as a chemical transmitter as the cholinergic nervous sytem. There are four components on the basis of which a cholinergic system in nervous tissues has been delineated: 1) acetylcholine; 2) cholinesterases that hydrolyze ACh into choline and acetic acid; 3) choline acetyltransferases that catalyze the transfer of the acetyl moiety from acetylcoenzyme A to choline; and 4) cholinergic receptors at which ACh binds to produce the physiological response.

During the past 50 years, several tissues without innervation (*e.g.*, human placenta, erythrocytes, and spermatozoa) have been shown to contain one or more components of the cholinergic system of nervous tissues. This gave rise to questions about the role of ACh in non-nervous tissues. Further, theories have been put forward that ACh may have functions other than chemical transmission even in nervous tissues. Therefore, the authors have attempted to collect information on selected tissues without innervation or tissues in which alternative functions for ACh have been postulated. It is anticipated that this review may shed light on the possible common role or roles for ACh. Alternatively, this review may bring into focus the gaps in our knowledge about the components of the cholinergic system in these tissues that prevent definite conclusions about the role of ACh in these tissues. For the purpose of this review, the cholinergic system in the nervous system is referred to as the nervous cholinergic system to distinguish it from cholinergic systems in other tissues, such as the placental cholinergic system.

A. Components of the Cholinergic System

1. Acetylcholine and Other Choline Esters. The brilliant investigations of Otto Loewi on "Vagustoff" in 1921 have provided evidence for the role of ACh as a chemical transmitter. Since then many choline esters other than ACh have been re-

ported to occur in animal nervous tissues, as well as in nonconducting or nerve-free organs (spleen, section IV A2; spermatozoa, section VII; placenta, section VIII) (14, 28, 29, 114, 213, 271, 396, 485, 486). ACh is synthesized *in vivo via* a coupled system involving an acetate-activating enzyme (acetyl-CoA-synthetase) and an enzyme that couples the activated acetate to choline. Transfer of the acetyl group from acetylcoenzyme A to choline is catalyzed by choline acetyltransferase. Carboxylic acids other than acetic acid and other analogs of choline do seem to enter the ACh synthetic pathway.

Acetone powder extracts of pigeon brain and squid ganglia can synthesize active choline esters if propionic acid or butyric acid is added to the medium in place of acetic acid (263). Choline acetyltransferase is by no means specific for choline but can acetylate many analogs of choline (44). Recently, many choline esters have been reported to occur in animal tissues. These include acetylcholine (97, 396), propionylcholine (14, 28, 29, 245), butyrylcholine (213), acrylylcholine and its analogs (114, 485, 486), γ-amino-butyrylcholine (271), and many other unidentified choline esters. Furthermore, recent investigations suggest that a "β-methylcholine" moiety may be formed in animal tissues by the decarboxylation of carnitine (319). Phosphotidyl-β-methyl choline is known to occur in animal tissues (23). However, it is not known whether β-methylcholine enters the ACh synthetic pathway. The possibility of the occurrence of lactoylcholine in animal tissues has been suggested by some investigators (343, 398). This suggestion is based on the pharmacodynamics of choline esters and atropine-like agents (381, 398), the naturally occurring choline esters, the specificity of cholinesterases (10, 279, 399, 400), and the specificity of choline acetyltransferase (44, 65, 263). Recently, it has been suggested that triethylcholine may enter the ACh synthetic pathway and the acetylated product may serve as a "false transmitter" (69). A knowledge of the specificities of the enzymes and their mechanisms in both steps in the synthesis of ACh is necessary before the quantitative aspects of the synthesis of other choline esters can be assessed.

The choline esters of carboxylic acids containing three carbon units (analogs and derivatives of propionic acid) are of considerable biological significance because acyl-coenzyme A's of these derivatives (242) occur in living tissues: Hydration of acrylyl-coenzyme A to α-hydroxypropionyl-coenzyme A (lactoyl-coenzyme A) has been reported to occur both in pigeon heart muscle preparations and in extracts of a propionate-oxidizing species of Pseudomonas (465). Acrylyl-coenzyme A can also be hydrated to β-hydroxypropionyl-coenzyme A by mammalian enoyl-coenzyme A hydratase (242, 357, 465). Conversion of propionyl-coenzyme A to acrylyl-coenzyme A involves β-oxidation and is analogous to the formation of α, β-unsaturated fatty acyl-coenzyme A catalyzed by acyl-coenzyme A dehydrogenase (242). Therefore, the conversion of propionyl-coenzyme A to acrylyl-coenzyme A, and the acrylyl-coenzyme A to β-hydroxypropionyl-coenzyme A and α-hydroxypropionyl-coenzyme A (lactoyl-coenzyme A) may occur in animal tissues. Propionic (CH_3CH_2COOH), acrylic ($CH_2{=}CHCOOH$), lactic ($CH_3CHOHCOOH$), and β-hydroxypropionic ($CH_2OHCOOH$) acids are closely related acids and there are biochemical mechanisms for the synthesis of their choline esters in animal tissues.

Unlike propionylcholine (14, 28, 29, 245) and acrylylcholine (114, 485, 486), lactoylcholine and β-hydroxypropionylcholine have yet to be shown to occur in nature. L-Lactoylcholine is hydrolyzed by both types of cholinesterases at rates comparable to those for ACh (10, 279, 399, 400). In a coupled system, containing acetyl-thiokinase, human placental choline acetyltransferase, and the necessary cofactors, the rate of synthesis of L-lactoylcholine is comparable to that of ACh at high substrate concentrations (65). Therefore, occurrence of

lactoylcholine in tissues is possible, especially under pathological conditions when lactate accumulates in tissues. It has yet to be shown that choline esters other than ACh occur in mammalian nervous systems.

2. Cholinesterases. Acetylcholinesterase, cholinesterase, and related enzymes and their occurrence in both nervous and non-nervous tissues have been subjects of many reviews and monographs (2, 204, 251, 328, 364, 424, 464, 488). Acetylcholinesterase is specific for the hydrolysis of ACh, which is the natural substrate for this enzyme in nervous tissue. Considerably more is known concerning its cytological distribution because of the availability of histochemical methods for its localization, and the use of irreversible and reversible anticholinesterase agents. The relative importance of the generally assumed function of acetylcholinesterase, destruction of ACh at the postsynaptic site, probably varies with the location and functional state of the nerve. The location of acetylcholinesterase at presynaptic sites of certain autonomic ganglia is not well understood. It is possible that presynaptic acetylcholinesterase protects the nerve terminals from depolarization by ACh. It is also possible that there may be presynaptic cholinergic receptors that are activated to cause feedback inhibition of ACh release. It is quite possible that the enzyme serves primarily to prevent accumulation at both pre- and postsynaptic sites of ACh liberated continually during the resting stage, and its role in the metabolism of ACh following transmission is secondary.

The natural role of cholinesterase in the blood plasma and other tissues is not well understood.

3. Choline Acetyltransferases. It is now known that the enzyme choline acetyltransferase catalyzes the synthesis of ACh from choline and acetylcoenzyme A in the nerve terminal. However, several questions about the biosynthesis of ACh in nervous or non-nervous tissues have yet to be answered. The nature of the enzyme *in situ,* its properties and intracellular location, and its functional relation to other metabolic systems of the cell have yet to be established. There seem to be multiple forms of the enzyme in various tissues, the significance of which is not well understood (182, 286, 484). Several aspects of choline acetyltransferase are summarized in excellent reviews by Hebb (180–183).

Lack of a suitable choline acetyltransferase inhibitor has hampered progress of research on this enzyme in nervous as well as non-nervous tissues. 4(1-Naphthylvinyl)pyridine and related compounds are used as inhibitors (IC50 about 10^{-6} M) of choline acetyltransferase (55–57, 438, 481–483). They are *trans* isomers and photoisomerize readily in solution to *cis*-isomers, which are poor inhibitors of choline acetyltransferase (481). During exposure to daylight, their use is thus limited with respect to obtaining reliable data *in situ* and *in vivo* pharmacological experiments. Sastry and collaborators (72, 74, 75, 385, 387, 389) have synthesized monohalogenoacetylcholines and studied their pharmacological activities. Among halogenoacetylcholines, chloro-, bromo-, and iodoacetylcholines (IC50 about 10^{-7} to 10^{-6} M) are strong specific inhibitors of choline acetyltransferase (187–189, 297, 391–393, 441). However, they are hydrolyzed by cholinesterases (71, 73, 369, 370, 386, 388). The tertiary analogs of halogenoacetylcholines are less potent inhibitors of choline acetyltransferase than the corresponding quaternary ammonium compounds (189, 369). Persson (341, 342) prepared 3-chloro-, bromo-, and iodoacetonyltrimethylammonium halides that inhibit choline acetyltransferase (IC50 about 10^{-5} to 10^{-4} M). They are not chemically stable, and their specificity for inhibiting choline acetyltransferase is not known.

5-Hydroxy-1, 4-naphthoquinone (juglone from the extract of walnut hulls) inhibits choline acetyltransferase (177), but the selectivity of this inhibitor is not established. Several naphthoquinones inhibit ACh formation in coupled-acetylcholine-synthesis using multienzyme systems (300), and several thiol reagents inhibit choline

acetyltransferase (365, 366). However, these reagents inhibit all enzymes containing sulfhydryl groups and are not specific for choline acetyltransferase. Acetylsecohemicholinium-3 inhibits choline acetyltransferase (95, 99), but it also inhibits the choline uptake system. Recently 2-benzoylethyltrimethylammonium has been introduced as a stable, selective, and specific inhibitor of choline acetyltransferase (64, 368). Although its pharmacology has not been fully studied, 2-benzoylethyltrimethylammonium and its tertiary analogs may become suitable inhibitors for *in vitro* and *in situ* studies.

4. Acetylcholine Receptor. The nicotinic receptor protein from fish electric organ was the first cholinergic receptor to be purified and isolated in milligram quantities (83, 109, 320). Cholinergic receptors have not yet been isolated from any non-nervous tissues.

B. Methodological Considerations

1. Acetylcholine. The most widely used and sensitive methods for the determination of ACh fall under the categories of bioassay and gas chromatography.

A. BIOASSAY. In bioassay methods, the sensitivities to ACh of (a) the leech longitudinal muscle, (b) the frog rectus abdominis muscle, (c) the longitudinal ileal muscle of the guinea pig, (d) the heart of *Venus mercenaria,* and (e) the cat blood pressure have been utilized for the estimation of ACh (89, 284). About 0.2 to 5 ng of ACh can be assayed depending on the tissue, the degree of separation of ACh from the tissue, and the size of the organ bath. By using superfusion techniques, the sensitivity of assay can be increased by about 100-fold. Using different bioassay preparations, which are sensitive to different biogenic amines and which are arranged as a cascade for superfusion, it is possible to assay for ACh as well as other biogenic amines in the same tissue extract simultaneously (344).

The bioassay method for ACh is relatively simple, cheap, and rapid. Although it is generally regarded as nonspecific, it can be made specific by selecting suitable preparations and by the use of specific blocking agents for other naturally occurring biogenic amines. The chief disadvantage of bioassay is that ACh cannot be identified during the assay procedure itself.

B. GAS CHROMATOGRAPHY. Acetylcholine and related quaternary ammonium compounds cannot be analyzed directly by gas chromatography because they do not exert significant vapor pressure. Therefore, they must be converted to volatile compounds stoichiometrically for analysis by gas chromatography. There are four chemical reactions that have been used for the analysis of ACh by gas chromatography.

The choline esters can be hydrolyzed by alkaline hydrolysis and the resulting acids can be analyzed by gas chromatography (87). Because the acid portion of the choline esters is analyzed, the method can be adapted for the analysis of a wide variety of choline esters.

In a second method, ACh is reduced and split to ethyl alcohol by potassium borohydride. The ethyl alcohol is estimated by gas chromatography (442, 443). This method is also applicable for analysis of propionylcholine and butyrylcholine. Values for ACh obtained by this gas chromatographic method are, in general, higher than those obtained by bioassay using guinea pig ileum, for unknown reasons. The presence of acetyl-*dl*-carnitine, acetyl-β-methylcholine, γ-aminobutyrylcholine, or γ-butyrobetaine methyl ester do not interfere in the estimation of ACh by this gas chromatographic method.

In the third method, ACh is converted into dimethylaminoethyl acetate by N-demethylation by benzenethiolate in butanone solution (165, 228–230). Conversion of quaternary ammonium compounds to tertiary amines by benzenethiolate ion was reported by Shamma *et al.* (422). This reaction is relatively selective in removing an N-methyl group at low temperatures. Shamma *et al.* (422) found that this reaction could not be used with esters. However, Jenden *et al.* (230) developed reaction

conditions in which an N-methyl group could be selectively removed from ACh without concurrent attack on the ester bond. This was achieved in anhydrous butanone at 80°C, using special precautions to remove reactive impurities in the benzenethiolate reagent. The resulting dimethylaminoethyl acetate is separated by solvent extraction from excess reagent and nonbasic reaction products and is estimated by gas chromatography. The detection limit for ACh would be 0.08 nmol or 14 ng. This reaction can be used for simultaneous estimation of ACh, propionylcholine, and butyrylcholine.

Pyrolysis of ACh chloride (or iodide) and related choline esters at high temperatures gives the corresponding 2-dimethylaminoethyl esters and methyl chloride (or iodide) (155, 403–406, 454). The resulting 2-dimethylaminoethyl esters can be analyzed by gas chromatography. This method is used for the estimation of ACh, acetyl-β-methylcholine, butyrylcholine, β-methylcholine, and choline. All of these compounds should be present as their halide salts in the samples for analysis. The halide anions of these compounds are strong nucleophiles at high temperatures to be split off as methyl halides.

By the above methods, amounts of ACh varying between 2 and 50 ng can be estimated. The sensitivity of the method is dependent upon the degree of separation and purification of ACh. Among the above gas chromatographic methods for ACh, methods using the chemical or pyrolytic N-demethylation have become popular. These gas chromatographic methods have been used for the estimation of ACh in mouse and rat brain (403, 404), human placenta (396), bull and human spermatozoa (28), guinea pig ileum (322), and in several other tissues (405). In tissue extracts containing high concentrations of choline relative to ACh, choline should be converted to butyrylcholine or another derivative to avoid interference of choline with the analysis of ACh. Gas chromatographic methods have also been used for the assay of propionylcholine in ox spleen and bull spermatozoa. Methods involving N-demethylation are not suitable for the analysis of choline esters (*e.g.*, lactoylcholine) if the N-demethylated derivatives are not stable.

Combined gas chromatography and mass spectrometry has been used for the identification and estimation of ACh, which is subjected to chemical or pyrolytic demethylation (229, 444). The demethylated choline esters are assayed using mass fragmentography by focusing on *m/e* 58, which is the most prominent fragment. This fragment corresponds to dimethylenimmonium ion $(CH_3)_2N{=}CH_2^+$. The gas chromatography-mass spectrometric methods for the assay of ACh are as sensitive as the bioassay methods. Although these methods have been used for the analysis of brain (229), spermatozoa (29), and spleen (240), the initial cost of equipment and subsequent maintenance is beyond the reach of the average laboratory. Therefore, these methods have not become as popular as simple bioassay and gas chromatographic methods.

c. MISCELLANEOUS METHODS. Several other methods for ACh and choline using fluorometric procedures, polarographic procedures, photometric techniques, and coupled enzyme reactions have been described. For details about these methods, recent reviews on this topic should be consulted (166, 167).

2. Cholinesterases. Several methods have been described for the assay of both cholinesterases using specific substrates and specific inhibitors. While ACh is a substrate for both acetylcholinesterase and pseudocholinesterase, specific substrates are available for these enzymes. Acetyl-β-methylcholine can be used as a specific substrate for acetylcholinesterase because it is not hydrolyzed by pseudocholinesterase (399). Butyrylcholine can be used as a substrate for the analysis of pseudocholinesterase (400). It is a very weak substrate for acetylcholinesterase. Each method is applicable for a specific situation based upon the amount of tissue available and the sensitivity required in the assay. In all

methods tissue homogenates can be used as the source of the enzymes.

In the most popular method, the hydrolysis of choline esters is determined by titrating the acids released during their hydrolysis using a Radiometer pH-stat titration unit. The details are described in published papers (231). This method has been extensively used in the analysis of tissue cholinesterases as well as for the kinetic characterization of purified enzymes (66, 67). A colorimetric method has been developed by Ellman *et al.* (111), in which thiocholine produced by hydrolysis of acetylthiocholine reacts with 5, 5-dithiobis-2-nitrobenzoate to yield the yellow 5-thio-2-nitrobenzoic anion. The absorbance corresponding to the amount of acetylthiocholine is measured at 412 mμ. This can be made specific for acetylcholinesterase and pseudocholinesterase by the use of specific substrates or inhibitors. However, this method is not suitable for studying the hydrolysis of different choline esters by cholinesterases.

A sensitive method is a differential radiochemical assay for acetylcholinesterase and pseudocholinesterase using ^{14}C-substrates (186). The differential rates of hydrolysis of 1-^{14}C-acetyl-β-methylcholine and 1-^{14}C-butyrylcholine are used to estimate acetylcholinesterase and pseudocholinesterase activities in tissues. The tissue homogenate and the ^{14}C-substrate are incubated for varying periods and initial linear velocities are obtained from rates of hydrolysis. As indicated previously, acetyl-β-methylcholine is not significantly hydrolyzed by pseudocholinesterase, while butyrylcholine is hydrolyzed by acetylcholinesterase at 12% of the rate of acetyl-β-methylcholine. Therefore, a correction is applied in the estimation of pseudocholinesterase by butyrylcholine. This method is of special value in estimating cholinesterases in small amounts of tissues (plasma 0.01 to 0.1 ml, tissue 10 mg). This method is sensitive but is expensive because it requires ^{14}C-labeled substrates.

A sensitive radiometric method, in which ACh is hydrolyzed by cholinesterases in Krebs-Ringer bicarbonate containing $NaH^{14}CO_3$, has been developed by Chiou (68). Experiments are conducted using a Warburg apparatus with 2-phenethylamine placed in the central well of the flask to trap $^{14}CO_2$ produced. At the end of the reaction, 2-phenethylamine with $^{14}CO_2$ is transferred to a counting vial containing scintillation cocktail and counted with a liquid scintillation counter. This method is very useful to determine cholinesterases bound to membrane fractions. The hydrolysis of any choline ester can be measured using this method because different cholinesters can be used as substrates in this reaction. The method is relatively inexpensive because radiolabeled substrates are not required for this assay.

Considerably more is known concerning the cytological distribution of cholinesterases since they can be localized histochemically by light microscopy using the thiocholine method developed by Koelle *et al.* (252, 253, 260, 261). Several excellent reviews have been published on histochemical localization of cholinesterases and the usefulness of these methods for the delineation of the functional anatomy of synaptic transmission (250, 251, 255). A new histochemical procedure that would approach in accuracy the degree of resolution afforded by electron microscopy has been achieved with the bis-(thioacetoxy) aurate ($Au(TA)_2$) method. However the $Au(TA)_2$ method is not as specific as the earlier copper thiocholine procedure (257, 259). Selective inhibitors of acetylcholinesterase do not readily permeate *in vitro* the small tissue blocks customarily used for conducting enzymatic histochemical reactions prior to embedding and sectioning for examination by electromicroscopy (258). Further developments of these exciting methods may be achieved in the future.

3. Choline Acetyltransferases. The final step in the biosynthesis of ACh is catalyzed by choline acetyltransferase. This step involves the following reaction: Choline + acetylcoenzyme A $\rightleftharpoons$ acetylcholine + coenzyme A. Acetylcholine formed in this reaction can be analyzed by a variety of meth-

ods. In the most common and popularly used methods, ^{14}C-ACh formed in the reaction is analyzed by a radiometric assay. In radiometric assays for choline acetyltransferase, the acetyl group of the substrate (acetylcoenzyme A) is labeled and during the course of the reaction some of this label is transferred to choline, where it can be recovered as labeled ACh. The amount of ACh formed in this way is calculated from the specific activity of the acetylcoenzyme A originally present. Two methods of providing labeled acetylcoenzyme A are: 1) from a coupled system; and 2) by adding labeled synthetic acetylcoenzyme A.

A. Formation of ACh by coupled synthesis. In the coupled system, labeled acetylcoenzyme A is formed in the reaction medium from labeled acetate and coenzyme A in the presence of suitable enzyme and cofactors. Then, labeled acetyl group from acetylcoenzyme A is transferred to choline in the presence of choline acetyltransferase.

Coupled assay systems have been in use for several years, and a number of different enzymes (including phosphate transacetylase (EC 2.3.1.8) and acetylcoenzyme A synthetase (EC 6.2.2.2)) with appropriate substrates have been employed for the formation of acetylcoenzyme A. The main advantage of this method is that acetylcoenzyme A is constantly regenerated as its acetyl groups are transferred to choline; thus it is possible to maintain the initial rate of synthesis in incubations at about 37°C for long periods (up to 60 min). The choice of the donor enzyme for the supply of acetylcoenzyme A is governed by the properties of the test enzyme. If choline acetyltransferase in brain extracts from a cold-blooded vertebrate species is being measured at an incubation temperature of less than 30°C, phosphate acetyltransferase is the suitable donor enzyme since it is active at these lower temperatures as well as at the higher temperatures suitable for avian and mammalian tissues (180, 182, 299). In employing coupled systems, it has been usual, until relatively recently, to measure the amount of ACh produced in a given period by bioassay. In 1963, Schuberth (409) published an account of a coupled system in which ^{14}C-acetate in the presence of acetylcoenzyme A, synthetase from yeast and coenzyme A forms ^{14}C-acetylcoenzyme A, which in turn yields ^{14}C-ACh. Other radiometric assays employing coupled systems have been developed since this pioneer work of Schuberth. Fonnum (127) has described an assay that can be used on either a micro- or a macroscale. It also utilizes 1-^{14}C-acetate and coenzyme A in conjunction with acetylcoenzyme A synthetase of pigeon liver for the formation of 1-^{14}C-acetylcoenzyme A. The 1-^{14}C-ACh formed is separated from other labels by precipitation with tetraphenylboron sodium (Kalignost). The precipitate is then washed, dissolved, and counted in a scintillation spectrometer. In another coupled method (298) tritiated acetylphosphate is employed as the initial substrate; in the presence of phosphate transacetylase and coenzyme A, labeled acetylcoenzyme A is formed; in the presence of choline acetyltransferase this in turn yields labeled ACh, which is separated either by high-voltage electrophoresis or on an anion exchange column and counted in a scintillation spectrometer.

B. Formation of ACh from acetylcoenzyme A and choline. Several authors have described methods in which synthetic labeled ^{14}C-acetylcoenzyme A was used as a substrate for the assay of choline acetyltransferase in tissues. The assay method varies very much from one laboratory to another (128, 141, 290, 407). This is important to remember since puzzling differences can often be traced to the conditions of the assay method. Other factors that affect the level of choline acetyltransferase activity in tissues are the methods of extraction and, in the case of brain tissue, of activation. It, therefore, is worth considering which factors are important for quantitative assays of choline acetyltransferase.

There are three sources of error in quantification of choline acetyltransferase in tissues (182). First, the incubation medium for

assay should be constituted so that only the concentration of the enzyme is rate-limiting in the formation of ACh. If the concentration of acetylcoenzyme A is insufficient, the full activation of the enzyme may not be observed. Second, the method of extraction should be such that all the enzyme in the tissue is sampled and is measured. Otherwise, what is being sampled does not truly represent the whole enzyme and the activity of the occluded enzyme escapes measurement. Third, the method of extraction may lead to the loss of choline acetyltransferase because of denaturation, which may not be equal in different fractions of tissue. These errors may vary from tissue to tissue. These errors are discussed in detail for nervous tissue in an excellent review by Hebb (182).

II. Occurrence of One or More Components of Cholinergic Systems in Organisms and Tissues without Nerves

Several tissues are known in which the involvement of the nervous system is either remote or absent (*i.e.,* certain portions of skeletal muscle, the blood and hematopoietic tissue, the placenta, and certain endocrine glands). In many autonomic effector organs (*e.g.,* heart, smooth muscle) ACh probably acts both in the transmission of parasympathetic nerve impulses and as a local hormone. The above tissues have been discussed for convenience at appropriate places. The structures considered in the present section are by known evidence devoid of nervous control.

A. Unicellular Organisms

1. Bacteria. Acetylcholine has been identified in a bacterium, *Lactobacillus plantarum* (445). Cells of *L. plantarum* produce ACh under optimum conditions at the rate of 1 nmol/min/mg of dry cells (482). This production is reduced by inhibitors of choline acetyltransferase.

The presence of a choline acetylating enzyme in the bacterium, *L. plantarum*, has been reported by Stephenson and Rowatt (445). Subsequently, several investigators have described the isolation and some properties of an extract obtained from this bacterium (90, 139, 407, 469). White and Cavillito (482) have partially purified the enzyme and studied its properties. In its kinetic properties this bacterial enzyme resembles the choline acetyltransferase from calf caudate nucleus. Both enzymes are stabilized by dithiothreitol and EDTA. The extracts differ in that the bacterial enzyme is more labile and is apparently more susceptible to conformational changes, which modify its response to inhibitors, styrylpyridines. The use of these intact bacterial cells as a test system *in vivo* with nonquaternary compounds is feasible, since similar degrees of inhibition are observed for 4(1-naphthylvinyl)pyridine using intact cells or extracts. The intact cell system cannot be used with the quaternary inhibitors of the pyridiniun type (*e.g.,* N-methyl-4(1-naphthylvinyl)pyridinium iodide) because of restricted permeability of the cell membrane to the charged species.

In 1953, Goldstein and Goldstein (146) described a strain of *Pseudomonas fluorescens* that was isolated from fermenting cucumber by enrichment culture with ACh as a sole source of carbon. This organism produces a cholinesterase, the increased synthesis of which can be induced by choline and choline esters.

The inducible cholinesterase produced by the Goldstein strain (146) of *P. fluorescens* has been purified to a state of electrophoretic homogeneity (276, 277). The enzyme resembles acetylcholinesterase in its substrate specificity and has a high affinity for ACh (K_m 1.4×10^{-5} M) and propionylcholine (2×10^{-5} M). The bacterial acetylcholinesterase reacts very slowly with tetraethylpyrophosphate (TEPP) and diisopropylphosphorofluoridate (DFP) but comparatively rapidly with ethyl N, N-dimethylphosphoramidocyanidate (tabun). Although it is resistant to physostigmine (10^{-3} M, no effect) it is very sensitive to prostigmine (IC50, 1.3×10^{-5} M), a property which has been observed in the cholines-

terases of certain lower animals (179). The reactions of bacterial acetylcholinesterase depend upon the ionic state of the groups in the enzyme whose pKa values are in the same range as those reported for other esterases, and both histidine and serine may be involved in its activities.

There are no studies on the role of ACh, choline acetyltransferase, and acetylcholinesterase in bacteria. Foust and Doetsch (119) have reported a study of the effects of several pharmacological agents on the motility of two photosynthetic bacteria: *Rhodospirillum rubrum* and *Thiospirillum jenense*. The former bacterium has bipolar flagellation, the latter has a flagellar fascicle at one pole. These experimental results are useful heuristically. The agents used in the above study include serotonin, local anesthetics, cholinesterase inhibitors, and cholinomimetics. All of these agents modify motility in some way, and most of them halt bacterial movement completely at concentrations in the vicinity of 10^{-3} M. The inhibition of movement by bacteria may be due either to cessation of flagellar movement or loss of coordination wherein the flagella remain motile but no propulsion occurs. The two bacteria may respond differently and a dose of atropine of about 10^{-3} M stops motility completely. Physostigmine and some other cholinesterase inhibitors inhibit motility in both bacteria; but neostigmine has no effect even after 48 hours of exposure. It is possible that quaternary ammonium compounds such as neostigmine do not penetrate to the site of action in these bacteria.

2. Protozoa. The presence of both ACh and an eserine-sensitive enzyme that promotes its hydrolysis in a species of *Paramecium* was reported by Beyer and Wense (22). According to Bülbring *et al.* (40) ACh and choline acetyltransferase are present in the motile flagellated protozoan, *Trypanosoma rhodesiense,* but not in the nonmotile unicellular organism, *Plasmodium gallinaceum.* They have speculated that ACh may be involved in rapidly moving protozoa like trypanosomes but not in sluggish ameboid movement.

Homogenates of the ciliated protozoan, *Tetrahymena gelii S.* hydrolyze ACh. This hydrolysis is blocked by 4×10^{-7} N physostigmine sulfate or DFP. The ciliary activity of the intact organisms is inhibited reversibly by the same agents at 10^{-3} M (417, 418). However, no ACh-hydrolysis is observed when homogenates of *Tetrahymena pyriformis* or *gilii W.* or two flagellated protozoans, *Polytoma uvella* and *Polytomella Caeca* are used (458). Hestrin's colorimetric method (200), which has been used in the above studies, is possibly not sensitive enough to detect the low rate of ACh hydrolysis by the homogenates of the latter species.

Little or no acetylcholinesterase and butyrylcholinesterase have been found in the homogenates of the amoeba, *Chaos chaos,* by manometric techniques (251). Manometric techniques are not sensitive enough to detect small amounts of cholinesterases; further investigations are necessary in this area using modern radiometric techniques.

In the acellular slime mold, *Physarum polycephalum,* there is acetylcholinesterase (302). According to Nakajima and Hatano (302), the ACh system may have a functional role in protoplasmic streaming in the plasmodium of the myxomycete. Alternatively, the enzyme may be held over from the previous flagellated stage in the life cycle although such a stage is missing in the authors' laboratory culture conditions. The effects of ACh and cholinesterase inhibitors on protoplasmic streaming of the above myxomycete are not reported by Nakajima and Hatano, and no report of the presence of an ACh system in the flagellated swarm cell stage of myxomycete is available. According to a subsequent study (206), ACh can modify protoplasmic streaming in another myxomycete, *Physarella oblonga.* The protoplasmic streaming in the plasmodium of this organism exhibits rhythmic reversal of the direction of flow. The durations of each phase of flow are equal and the sum of the two durations that constitutes one cycle is very precise within the time limits of experimental observations. Both ACh and physostigmine prolong

the duration of each cycle when added separately to the culture. Combination of these two agents produces the same effect faster and with more intensity, but with shorter duration of action. The rapid agonist action of ACh is possibly followed by its antagonist action in the presence of the cholinesterase inhibitor due to the accumulation of high levels of ACh. These observations suggest a role for ACh in protoplasmic streaming.

B. Multicellular Organisms

1. Nonmammalian Organisms. The role of ACh in the ciliary movement of mussels, *Mytilus* and *Anodonta,* has been studied by some investigators, and a summary of their studies is included in this section. The role of ACh in the ciliary movement of the frog esophagus and the rabbit trachea are included in sections III C1 and III C2.

A. GILL PLATES OF MYTILUS EDULIS. Although it has been shown in other tissues that ciliary movement is independent of the nervous system and cannot be influenced by local anesthetics, a tissue is required in which the ciliary movement could be studied in the absence of nerve fibers. The gill plates of the mussel, *Mytilus edulis* provide such a tissue and Bülbring *et al.* (39) have studied ciliary movement in them.

A description of the gill plates is included in the review by Burn (48). Gill plates do not contain nerves or muscles. Each plate consists of several V-shaped filaments next to one another and loosely connected by means of ciliated discs. In transverse section each filament has a single layer of cubical cells around a central lumen. Tracts of the cells, along the long axis of the filament, bear large numbers of actively beating cilia, those of different tracts being histologically distinct and differing slightly in their mode and frequency of beat (48). Large amounts of mucus are produced by the tissue.

Two methods of measuring ciliary activity can be used with this preparation: one by measuring the rate of transport of graphite particles across the surface of the gill plate and the second by measuring the rate of the beat of the cilia with the aid of a stroboflash. The detailed methods are described by Burn (48). Acetylcholine increases ciliary movement when applied in low concentrations and decreases it in higher concentrations (Table 1). Physostigmine inhibits cholinesterase and the application of eserine should therefore cause accumulation of ACh in the tissue. Physostigmine behaves like ACh; in low concentrations it increases the ciliary activity and in higher concentrations decreases it. This indicates that ACh present in the gill plate influences ciliary activity. This conclusion is supported by the effect of *d*-tubocurarine, an antagonist of ACh, which decreases the rate of ciliary movement in all concentrations, and its effect is reversible. *d*-Tubocurarine does not affect the secretion of mucus.

Gill plates contain 3.8 nmol of ACh chloride per g of fresh tissue when analyzed by bioassay. They also contain acetylcholinesterase, which hydrolyses about 32 to 33 μmol of ACh per g per hour. This enzyme does not have significant effects on the hydrolysis of benzoylcholine and butyrylcholine. Acetone-insoluble powders prepared from gill plates synthesize 8.5 and 15.3 nmol/g/hr in the presence of acetate and citrate as acetyl donor groups, respectively, when analyzed by the coupled synthetic method. Therefore, gill plates contain choline acetyltransferase.

Ciliary movement, like the contractions of rabbit auricles, is regulated by ACh synthesized locally. Various types of autonomous rhythmic activity are controlled by a fundamentally similar mechanism in which ACh is involved. Since the gill plates are without nerve fibers, they provide an example of a tissue in which ACh functions as a local hormone independently of its usual role as a transmitter of nerve impulses.

Epinephrine has a powerful accelerating action on ciliary movement, and the acid saline extracts of the gill plates contain an epinephrine-like substance, the action of which is abolished by veratramine. This action of epinephrine in potentiating the effect of ACh is well known and several

TABLE 1

Effects of various pharmacological agents on ciliary movement in Mytilus edulis[a]

Drug	Dose	Transport of Particles (% change)[b]		
		Frog esophagus	Rabbit trachea	Gill plate of Mytilus
	g/ml			
Physostigmine sulfate	10^{-6}			+
	10^{-5}	+41	+65	
	10^{-4}	+95	+98	
	2×10^{-4}	+39	Movement stopped	
	4×10^{-4}	−11		
Acetylcholine	10^{-7}			+10%
	10^{-6}			−16%
	10^{-5}	+50	+27	
	2×10^{-5}		+33	
	5×10^{-5}		−10	
	10^{-4}		−33	
Atropine sulfate	10^{-6}	−41	−55	+12[c]
	10^{-5}			+ 7[c]
	10^{-4}			+ 8[c]
	10^{-3}			−20[c]
d-Tubocurarine	10^{-6}	−41	−43	−2
	10^{-5}		Movement stopped	−17
	10^{-4}		Movement stopped	−19
	10^{-3}			−26
Cocaine	10^{-4}		No effect	
	10^{-3}		No effect	
	10^{-2}		No effect	
	2×10^{-2}		No effect	

[a] Summarized from Burn (48) and Burn and Day (49).

[b] +, acceleration; −, slowing of ciliary movement.

[c] In the presence of exogenous acetylcholine all values are reduced irrespective of acceleration or retardation.

examples have been discussed previously (203). The effect of epinephrine on ciliary movement in *Mytilus* may be another example of potentiation of ACh, but such a suggestion is entirely speculative in the light of present knowledge.

All of the above observations led to a proposal by Bülbring *et al.* (39) and Burn (46) that ACh functions as a local hormone in the maintenance of rhythmic movement of cilia on the gill plates of *Mytilus edulis*. This proposal is supported by convincing evidence that ACh, cholinesterase, and choline acetyltransferase are present in the gill. The major assumption is that the gill of the mussel is free from nerve fibers. This assumption has since been shown to be an oversimplification. The cilia on the gill plate of *Mytilus* can be distinguished into several types based on anatomical and functional criteria (203, 435). They also respond quite differently to changes in the electrolyte content of the medium (153). The neural and humoral control of ciliary motion in this group of organisms has been reviewed by Kinosita and Murakami (246), and the evidence reviewed by these authors can be summarized as follows. Each gill filament is covered by five groups of ciliated cells: one frontal, two frontolateral, and two lateral. In addition, pads of ciliated cells appear on the lateral surface of the gill. The

cilia of these pads are active when the filaments are separated; but when two filaments adhere to each other side by side, these pads form a ciliary junction and become inactive. The lateral cilia are controlled by the branchial nerve since they stop moving when the gill is isolated or when the branchial nerve is sectioned, and electrical stimulation of the nerve causes an increase in their rate of beating. Other cilia, however, remain active after isolation of the gill filament. It is likely that the increase in ciliary activity due to ACh observed by Bülbring *et al.* (39) was due to stimulation of the inactive lateral cilia, although other cilia may also be affected. 5-Hydroxytryptamine is also effective in stimulating ciliary motion in this organism and has been shown to be present in the tissue together with its associated enzymes for biosynthesis and inactivation.

5-Hydroxytryptamine (serotonin) influences the ciliary activity of *Mytilus* (1), and it is assumed to mediate the higher level control of the ciliary activity through the branchial nerve activity. According to Aiello and Guideri (1), the cholinergic system may be involved in nervous control, but serotonin is the more immediate agent in the regulation of ciliary motion. According to Gosselin *et al.* (149), serotonin may be the local hormone rather than ACh as proposed by Bülbring *et al.* (39). In addition to the presence of serotonin and its associated enzymes, Gosselin *et al.* (149) have reported increases in O_2 consumption, glycogenolysis, and glycolysis due to serotonin, and ACh has opposite effects.

B. GILLS OF *ANODONTA CYGNEA CELLENSIS*. As with *Mytilus*, recent evidence suggests that the ciliary activity in the gills of *Anodonta* is controlled by ACh at the tissue level (274).

Thermal resistance acclimation occurs in the ciliary activity in the gills of the fresh water mussel, *Anodonta*. This effect is also observed when isolated surviving gills are exposed to different environmental temperatures for 1 to 3 days (272). Similar resistance of acclimation occurs in ciliated antennae of gastropods (351). These observations suggest that temperature acclimation of the function would be the result of compensatory changes in the control mechanism of the respective function (275). If ACh and 5-hydroxytryptamine participate in the control of the ciliary activity, their systems should also participate in resistance acclimation.

Warm acclimation of the isolated gills for 20 to 72 hours prolongs the thermal resistance time (100%) of the activity of frontal cilia at 39°C (273). When measured in the presence of ACh (10^{-3} g/ml, 5.5 mM), the thermal resistance time is more than doubled. The effect of 5-hydroxytryptamine (2.5×10^{-3} g/ml as creatinine sulfate, 6.3 mM) is small, causing an increase of about 25% in the thermal resistance. The above effect of ACh on thermal resistance time is abolished by atropine. Acetylcholinesterase determinations and ACh assays show an increased turnover of ACh in the warm acclimated gills. Therefore, thermal resistance acclimation of the ciliary activity in the gills involves compensatory changes in the ACh system controlling the ciliary activity at the tissue level. The problem of physiological regulators of ciliary motion has been discussed in detail by Gosselin (148). The control of ciliary motion in mussels is far from resolved and a clearer definition of the role of the cholinergic system in the gill of these organisms must await further studies. The same may be said for the role of ACh in respiratory tracts of higher animals (section III C).

2. Mammalian Organisms. The non-nervous tissues of several mammals have been investigated for components of the cholinergic system. Several of these tissues are discussed in other sections of this review.

A. RED BLOOD CELLS. The components of the cholinergic system in erythrocytes and platelets of several species are discussed in sections VI A and VI B.

B. SPERMATOZOA. There is evidence for the existence of ACh, choline acetyltransferase, and acetylcholinesterase in the spermatozoa of several mammals. It has been

proposed that the cholinergic system in the spermatozoa maintains sperm motility. These aspects are discussed in detail in section VII.

C. PLACENTA. Although there are gaps, more work has been reported on the delineation of the components of the cholinergic system in human placenta. This is discussed in section VIII A. The possible existence of components of the cholinergic system in animal placentae and variation from species to species are discussed in sections VIII B to E.

D. ALLANTOIC MEMBRANE. There is an electrical potential across the allantoic membrane of the rabbit and there is also a marked difference in ionic composition between the allantoic and exocoelomic fluids (94, 97, 266). The allantoic membrane contains both acetylcholinesterase and choline acetyltransferase. The electrical potential across the allantoic membrane is sensitive to ACh and carbamylcholine, a sensitivity which can be altered or abolished by anti-acetylcholinesterases. The addition of ACh to the exocoelomic fluid produces a marked increase in the electrical potential whereas the opposite response is elicited by the addition of ACh to the allantoic fluid.

Acetylcholinesterase is present in the endodermal layer of the membrane. The only layer of cells with interlocking cell membranes and desmosomal contacts is the endotherm (52). Therefore, it is possible that the mechanisms associated with membrane potential may reside in this layer (52, 266). The role of ACh in basic mechanisms associated with membrane excitability of the allantoic membrane have yet to be delineated. It is also not known whether there is stored ACh that is released on excitation from this tissue, or whether ACh is utilized as it is synthesized.

III. Occurrence of One or More Components of Cholinergic Systems in Tissues in Which Their Involvement in the Nervous System is Remote

Several tissues are known in which the involvement of the cholinergic system is remote. The tissues contain one or more components of the cholinergic system. Except in cilia, the occurrence of only cholinesterases is known. The role of these components is not known.

A. Certain Portions of Skeletal Muscle

1. Musculotendinous Junctions. The relatively high concentration of acetylcholinesterase at the *musculotendinous junctions* of the individual fibers of striated muscle was first recognized by Couteaux (85). It has been confirmed and studied in detail in several mammalian species by Gerebtzoff (135, 137, 138) and Coers and Durand (82), and in the seahorse, *Hippocampus,* by Couteaux (86). According to Schwarzacher (412–415), the myotendinous enzyme consists entirely of acetylcholinesterase, and, in the rat and cat, the activity at each junction is approximately 20 to 30% of that in the corresponding neuromuscular junction. Its localization is restricted to the surface membrane at the end of the muscle fiber, where no innervation has ever been shown in mammals. According to Gerebtzoff (138), the concentration of the enzyme is unaffected by muscular denervation, and is actually increased by tenotomy.

The functional significance of the acetylcholinesterase at this location is unknown. The existence of choline acetyltransferase and ACh at this location is also not known. It cannot readily be dismissed as an evolutionary vestige reflecting earlier terminal innervation (251), as noted in many lower vertebrates, since in species of the latter group both terminal innervation and characteristically distributed myotendinous acetylcholinesterase have been noted concurrently (285). Unlike the neuromuscular junction, the myotendinous junction is not depolarized by ACh alone or in combination with neostigmine (414, 415). The enzyme may be associated with the endoplasmic reticulum (108), which is highly organized at the musculotendinous junction (285) and bears a definite resemblance to the acetylcholinesterase staining pattern (251).

2. Sarcolemma and Sarcoplasm. In sev-

eral species, the noninnervated portions of the sarcolemma and adjacent endomysium contain little or no acetylcholinesterase (254). However, there is evidence by histochemical methods that these areas in the tail muscle of the guppy (*Lebistes reticulatus*) and goldfish (*Carassius auratus*) contain acetylcholinesterase (283). According to some investigations (11, 301), in both of these species the level of cholinesterase in the muscles is extremely high and was identified as a typical acetylcholinesterase (270). In other fish, such as the beram (*Abramis brama*), the total acetylcholinesterase and its histochemical distribution do not differ significantly from the usual findings in striated muscle (283). The significance of this observation is obscure at present. It may represent phylogenetically a transitional stage, since during embryonic development in rats (270) and birds (136) acetylcholinesterase is distributed diffusely through the muscle fibers, then appears to be more concentrated at the sarcoplasmic membrane before assuming the specific sites of localization described above. Electron miscroscopic studies indicate the presence of numerous motor endplates along the muscle fibers of the goldfish (285).

In addition to the discrete staining for acetylcholinesterase at the sites discussed above, faint staining is generally present also throughout the sarcoplasm (17, 18). The possible specific localization of the enzyme at a sarcoplasmic site is suggested by the report of Barrnett and Palade (16), presumably due to a cholinesterase at the swelling of the thick elemental filaments at the M bands in both striated and cardiac muscle of the rat. Additional staining for cholinesterases, although of less certain enzymatic specificity, was observed in mitochondria, in round sarcoplasmic bodies, in contraction bands in the area of the Z bands, and in the sarcoplasmic reticulum. The above authors have indicated the possibility that the cholinesterase of the M bands may be identical with the myosin-cholinesterase found predominantly with L-meromyosin (466). During the growth of the chick embryonic skeletal muscle in tissue cultures, no endplate-like structures develop, but an acetylthiocholine-splitting enzyme accrues in the cytoplasm, particularly in the region of the Z line (112). According to Denz (96), the diffuse staining of myofibrils is due chiefly to aliesterase. Hence, the exact identities of the cholinesterases in these various sarcoplasmic locations, as well as their functional significance, are still obscure.

3. Miscellaneous Sites. Additional sites of staining for acetylcholinesterase in skeletal muscle that have been noted by several authors and were summarized by Beckett and Bourne (17, 18) and Koelle (251) include 1) structures made up of parallel gutters arranged as palisade, or "cake frill" around the muscle fiber, 2) parallel gutters, either parallel with or perpendicular to the long axis of the muscle fiber, and 3) spiral gutters wound around muscle fibers. The functional significance of all the structures is obscure.

B. Mucocutaneous Membranes

Frog skin contains cholinesterases that, on the basis of the relative rates of hydrolysis of various substrates, are probably largely nonspecific cholinesterases. Most of the activity is localized in the tela subcutanea (248). Examination of synthesis and hydrolysis of ACh by mammalian skin will be interesting in view of the regenerating capacity of epithelial layers of skin and the role of ACh in regeneration (section IV E). However, nothing is known about choline acetyltransferase and ACh in the skin.

C. Cilia

The role of ACh in ciliary movement in nonmammalian organisms (*Mytilus edulis, Anodonta cygnea cellensis*) has already been discussed in sections II B1A and II B1B. The role of ACh in ciliary movement in the respiratory tracts of amphibia and mammals has been demonstrated (48, 201). They are summarized in this section.

1. Frog Esophagus. A convenient preparation can be obtained from the frog esophagus for measuring movement of particles by cilia. The esophagus from a pithed

frog can be opened dorsally and pinned out so as to furnish a flat surface on which to observe the transport of particles by cilia. Selection of the particles, experimental preparation, and conditions of experiment were described by Kordik *et al.* (262) and Burn (46). The rate of transport of particles can be measured per 100 seconds or a minute by tuning the movement of each particle through a fixed distance (*e.g.*, 8 mm).

Acetylcholine in low concentrations accelerates the rate of particle transport and the rate is greatly reduced by atropine and *d*-tubocurarine. Physostigmine sulfate increased the rate in low concentrations but depressed it in high concentrations (Table 1).

2. Rabbit Trachea. Trachea from a freshly killed rabbit taken from below the larynx to the bifurcation can be removed, opened along the middorsal line, pinned on a cork mat, and used for measurement of the transport of charcoal particles by cilia. The details of the preparation have been described by Burn (46).

Rabbit tracheal mucous membrane contains 1.45 to 2.65 μg of ACh/g of tissue when analyzed by different bioassay preparations (frog heart, frog rectus, guinea pig ileum, and cat blood pressure). Acetone-insoluble powders prepared from the rabbit have choline acetyltransferase activity of 40 μg of ACh synthesized per g of powder per hour, although it is lower than that in the rabbit brain (680 μg of ACh/g/hr). Homogenates of tracheal mucous membranes hydrolyze ACh at a rate of about 121 μmoles/hr/g of tissue. They hydrolyze acetyl-β-methyl choline but not benzoylcholine.

The pharmacological experiments with the cilia of the rabbit trachea are consistent with the view that ciliary movement is controlled by the production of ACh, which when produced maintains the rhythmic activity (46). Low concentrations of exogenous ACh increase rhythmic activity and high concentrations depress the rhythmic activity (Table 1). The accelerating action of physostigmine has only one likely explanation, namely, that it is due to the inhibition of cholinesterase, and as a result ACh accumulates in greater concentration than before. Thus the action of physostigmine indicates that the ciliary movement is controlled by the production of ACh.

The ciliary movement is abolished by atropine and also by *d*-tubocurarine. These substances share the property of antagonizing the action of ACh, but only atropine depresses the secretion of mucus.

All of the above observations, together with the presence of ACh, choline acetyltransferase, and acetylcholinesterase in the tracheal mucous membrane are consistent with the view that ciliary movement is controlled by the production of ACh.

Both effects of ACh are seen when it is itself applied to the mucous membrane of the rabbit trachea. The lower concentrations accelerate the movement of the cilia, while the higher ones depress the movement (Table 1). It is questionable whether ACh ever produces inhibition in peripheral tissues. So far as ciliary movement in the mucous membrane of the trachea of the rabbit is concerned, there is no doubt of this. The same 2-fold effect is also to be observed with physostigmine, from which it can be concluded that ACh synthesis in the membrane can proceed so far that, if the acetylcholinesterase is completely inhibited, the accumulation of synthesized ACh can block the receptors on which it acts.

3. Human Respiratory Epithelium. Explants from punch biopsies of human tracheal and bronchial ciliated epithelium, when cultured in a physiological medium, curl up to form rotating globes that can be employed for the study of cholinergic and anticholinergic drugs on ciliary activity (88). These globes have cilia on their outside surface. Acetylcholine increases ciliary activity and thereby enhances the rotary activity of these ciliated globes. Physostigmine potentiates the effect of ACh on the rotatory motion of the globes. Atropine has a short-lasting stimulatory effect followed by progressive reduction of rotatory mo-

tion. The depressant action of atropine can be counteracted by ACh. These observations indicate that ACh plays a key role in initiating and maintaining the contractile mechanism of ciliary motion. However, there is no information on the synthesis of ACh by these globes.

IV. Occurrence of One or More Components of Cholinergic Systems in Tissues in Which Their Involvement Is Delineated into Nervous and Non-nervous Components

There are several tissues in which one or more components of the cholinergic system as well as nerve elements are present. In several cases, a neuronal role has been postulated for ACh in these tissues. A non-neuronal role is not completely eliminated in these tissues. Further investigations are necessary to establish the significance of the components of cholinergic systems in these tissues.

The question arises concerning the identity of the structures in which ACh is synthesized—in nerves, or in non-nervous tissue. Ciliary movement is generally believed to be autonomous in vertebrates (48, 201). Moreover, in the preparation of the frog esophagus system, the central nervous system is destroyed, and the ciliary movement is observed to be vigorous for many hours. There is no difference in ciliary movement, or in the action of drugs on it, whether the esophagus is left *in situ* or is excised (46). In the preparation of the rabbit's trachea (section III C2), the tracheal mucous membrane is isolated from the body and there is no circulation in it. When examined histologically, the mucous membrane contains no ganglion cells, but there are a few nerve fibers in the submucosa that may be sensory fibers and which are severed from the center during observations. It is probable that these divided nerve fibers could be the source of the ACh that maintains the ciliary movement, especially in view of the fact that cocaine hydrochloride in a concentration as high as 1 in 50 has no effect whatever on ciliary movement. Therefore, ACh is probably synthesized in tissue that is not nervous; the action of ACh in promoting ciliary movement in the tracheal mucous membrane is an example of a property distinct from that of a humoral transmitter, namely the property of a local hormone.

The candidacy of ACh as a local hormone in the control of ciliary motion of vertebrate respiratory tracts is often rivaled by serotonin. The proposal that ACh is involved in the regulation of ciliary activity and mucous flow in rabbit trachea (46, 262) is emulated by a similar suggestion for serotonin (90, 267). The interrelationship of these two agents and their respective biochemical or cellular accessory components have not been investigated systematically. The anticholinesterase activity at high concentrations and activation of the same enzyme at low concentrations of serotonin have been known for a long time (113). Although the functional significance of such relations between cholinergic and serotonergic systems is uncertain, their existence does indicate the rationale for further investigation. Moreover, evidence supporting the functional significance of ACh must also explain the rivalry by serotonin. ACh has also been implicated as a local hormone in the maintenance of rhythmicity in smooth muscle and cardiac muscle.

A. Smooth Muscle

1. Intestine. It has been known for a long time that intestinal smooth muscle will release ACh if incubated in eserinized solutions (98, 120, 121, 232, 334, 335, 402). Several of these studies were conducted using guinea pig ileum. The site of ACh release in these studies is controversial as to whether all ACh is released from the nerve elements or whether part of it is released from the smooth muscle. According to Feldberg and Lin (120, 121), part of the ACh released from the rabbit and the guinea pig intestine has a non-nervous origin. There are several studies that indicate that a major fraction of ACh is released from Auerbach's plexus. There is a direct

relationship between the ACh content and the degree of development of Auerbach's plexus, which suggests that the ACh released is of nervous origin (98). There are similarities in the effects of ions and drugs on the release of ACh from the nerve and its release from the intestine, which also suggests that ACh is of nervous origin. Mechanical denervation of the longitudinal ileal muscle of the guinea pig eliminates 98 to 99% of the stored ACh, 94 to 95% of the resting output of ACh, and 87 to 92% of the output in response to electrical stimulation (335).

The longitudinal muscle of the guinea pig ileum contains about 120 nmol of ACh/g of wet tissue (321, 322). In about four days of cooling at 4°C under anoxia, about 16 nmol of ACh/g of wet tissue remained in the tissue. This ACh does not seem to be available for release by electrical stimulation. All of these observations indicate that the major fraction of ACh is formed and stored in the Auerbach plexus. They do not completely exclude the possibility that some ACh is formed in the smooth muscle and is released at a constant rate.

There seem to be two components of ACh in the smooth muscle; the first and the major component is that ACh which is stored in Auerbach plexus and released by electrical stimulation, and the second component is that which is formed and directly released from the smooth muscle and is not influenced by electrical stimulation. Several cell types are now known that release ACh into the medium when incubated in a suitable medium (see under fibroblasts, section V A; spermatozoa, section VII). The level of the minor component of ACh in the smooth muscle is comparable to that found in other cell types that do not have anatomical storage compartments for ACh. It is necessary to know the turnover rates of these two components of ACh before a definite conclusion can be made. It is possible that the minor component may have a higher turnover rate in smooth muscle.

2. *Spleen.* Although detailed investigations of the various components of the cholinergic system in the spleen are not available, ACh and propionylcholine have been detected in ox spleen (14). Gardiner and Whittaker (132) have identified the ox spleen propionylcholine by infrared spectroscopy. These authors have measured total ACh-like activity on the frog rectus abdominis muscle and found that the total activity corresponded to 1.6 nmol/g of tissue. According to their estimates, ox spleen contains as much propionylcholine as ACh. According to more recent work in which choline esters from the ox spleen have been separated by high-voltage electrophoresis and estimated by gas chromatography, ox spleen contains 0.24 to 0.34 nmol of ACh/g of wet tissue and 0.46 to 0.79 nmol of propionylcholine/g of wet tissue (245). These values for ACh in ox spleen are about equal to those reported by Gardiner and Whittaker (132). The spleen of the sheep also contains 0.32 to 0.44 nmol of ACh/g of wet tissue and 0.14 to 0.23 nmol of propionylcholine/g of wet tissue (245).

The site of synthesis and the function of ACh and propionylcholine in the spleens of oxen and sheep remain obscure. The relative contributions of cholinergic nerves and the hemopoietic tissue to the total ACh and propionylcholine contents are not known.

The specificity of choline acetyltransferase in the spleen is not known. Propionylcholine can be synthesized *in vitro* from propionyl-coenzyme A and choline by brain choline acetyltransferase (348). Propionic acid and propionyl-coenzyme A occur in ruminants (347). Acetic acid and propionic acid are produced by bacterial fermentations in the rumen of the sheep where these acids amount to 57% and 27% respectively. It remains to be seen whether propionylcholine occurs in other ruminants as well. If this proves to be true, the occurrence of propionylcholine might be of interest in taxonomy.

B. Cardiac Muscle: Some Aspects

Acetylcholine has a stimulant action on the cardiac mechanism as well as an inhibitory action (47). The direct effect of initi-

ating contraction in quiescent cardiac muscle is not seen normally, but has been demonstrated in a variety of abnormal conditions, as when the auricles have been arrested by quinidine or by proguanil, or by cooling, or by allowing them to beat in Locke's solution until the contractions cease. A stimulant effect is, however, seen normally in the heart-lung preparation when a stream of impulses is sent into the auricular tissue under the influence of ACh, for this stream of impulses whether electrically induced or initiated by aconitine then causes fibrillation.

The importance of this stimulant or excitatory action of ACh for the cardiac mechanism is emphasized by the presence of the choline acetyltransferase system in cardiac muscle, and by the striking parallelism which was found between rhythmic activity and the activity of this system (47). When isolated rabbit auricles are suspended in an organ bath, they cease to contract after a certain period of time. Choline acetyltransferase activity in the stopped auricle is about one-fifth that in the beating auricle. Acetylcholine synthesizing power of restarted auricle increases again by about four times. Therefore, there appears to be a relationship between the synthesizing power and functional state of the auricle.

Acetylcholine depresses the rhythmic activity and also the synthesis in freshly excised auricles; when the auricles cease to contract in the bath, the addition of ACh causes the contractions to begin again; similarly, when the auricles cease to contract in a bath, synthesis in these articles is low. After the addition of ACh, the auricles resume contractions, choline acetyltransferase synthesis increases to a normal value. Therefore, it seems that at every stage the rhythmic activity is related to synthesis of ACh in the tissue.

When the auricles are cooled to 20°C or less, their electrical and mechanical activity ceases. However, there remain very small action potentials at the pacemaker which are not propagated. Addition of ACh as low as 10^{-9} M restores the electrical and mechanical activities (288). In the presence of added ACh, the small action potentials are propagated and the normal activity of auricles is restored. When ACh is washed, only small pacemaker action potentials remain. The choline acetyltransferase activity in the pacemaker activity is about 2.7 times higher than in the remainder of the tissue. Although low, the ACh synthesized in the auricular tissue may be adequate for maintaining the small pacemaker action potentials.

Rabbit auricles contain many nerve fibers, whereas the ventricles contain very few (294). However, 65 to 70% of ACh in the perfused heart is derived from the ventricles (47), which makes it improbable that ACh is formed in nervous tissue only.

An interesting problem is how to reconcile the stimulatory action of ACh in maintaining the beat with its well known inhibitory effect. The action of atropine is of interest in this regard. The inhibitory effect is promptly abolished by atropine, whereas the normal rhythmic contractions of the isolated auricles are only reduced and arrested by atropine after a prolonged application. Atropine, however, prevents ACh from causing the beat to begin again when once it has stopped, so that while the stimulant effect of externally applied ACh is sensitive to atropine, that of endogenously produced ACh is not. The effect of the ACh which is liberated by the beating heart either in the heart-lung preparation or when perfused with Locke's solution is also sensitive to atropine, so that if we regard this ACh as endogenously produced, then at the time it is playing its part in maintaining the rhythm of contractions it is unaffected by atropine, but when later it is set free in the blood or perfusion fluid it is then blocked by atropine. Any comprehensive hypothesis must take account of these observations (47).

The inhibitory action of vagus stimulation and of ACh is accompanied by, and presumably due to, a state of hyperpolarization, while, on the other hand, a stimulant action of ACh is accompanied by a depolar-

ization. The ACh might, however, not cause the depolarization itself but effect a change whereby a subthreshold depolarization becomes a threshold value. Here again a comprehensive hypothesis must reconcile these apparently opposite actions.

Some observations have raised questions concerning the function of the vagus nerve in the heart-lung preparation. When both vagi had been cut four days previously, the spontaneous rate of the heart-lung preparation was much lower than when the vagi had not been cut (51). The disappearance of vagal control did not result in a more rapid discharge of impulses from the pacemaker but in a much slower discharge. This finding suggests that the function of the vagus in relation to the heart is not purely inhibitory. This suggestion is of course of great interest and requires investigation.

The relationship of the nervous tissue in the heart to the conducting and muscular tissue presents another problem. The evidence points to non-nervous tissue rather than to nervous tissue as the site of the formation of a major part of ACh. If the heart beat is myogenic and is not transmitted by nerve fibers, it is necessary to discover the function of the nerve fibers, so abundantly present in the auricles (47).

Thus, many more questions remain to be answered. When more information is available, the hypothesis that the rhythmic contractions of the heart are maintained by endogenous formation of ACh can be further evaluated. This hypothesis, however, is useful in helping to make new observations as a result of which the cardiac mechanism may be better understood.

Although direct experimental evidence is lacking, several observations indicate that ACh may play a role other than chemical transmission in embryonic heart or myocytes. The appearance of acetylcholinesterase in cardiac muscle cells precedes innervation by the vagus nerve in chick, rabbit, and rat hearts (331). Cholinesterase has been found in association with rough surfaced endoplasmic reticulum of the fetal rabbit cardiac myocyte (164). It has been proposed that acetylcholinesterase is involved in differentiation of cardiac muscle cells and in the appearance of automaticity in developing animals (331). There is no experimental evidence for these proposals. Considerably less information is available about choline acetyltransferase and ACh-like substances in the embryonic heart before the development of nerves. The information we do have about ACh synthesizing capacity is contradictory. It has recently been reviewed by Pappano (331).

It is possible that ACh in embryonic heart and myocytes may play a role which is different from chemical transmission, as has been proposed for neuroblasts (see section V B). ACh may be involved in the release of trophic factors from myocytes, which may regulate the biochemical development of postganglionic cholinergic nerves. The influence of effector cells on the nature of neuronal input to them during differentiation has been reviewed by Bunge *et al.* (43).

C. Cornea

The corneal epithelium is considered to have very high concentrations of ACh. According to von Brucke *et al.* (468), there is 100 to 200 μg of ACh/g of corneal epithelium in both rabbit and cow eyes. According to Williams and Cooper (487), cow eyes contain 40 μg of ACh/g of tissue. Although corneal epithelium is rich in nerve endings, these high concentrations of ACh are not in accord with the level of ACh that is usually found in junctional tissue where it serves as a neurotransmitter (487). For example, in autonomic ganglia, the concentration of ACh ranges from 6 to 44 μg/g of tissue. Therefore, it is possible that ACh may have two roles in cornea, both neuronal and non-neuronal functions. Among the non-neuronal functions, ACh has been studied as a regulator of water and ion transport.

1. ACh as a Sensory Mediator. Local denervation of rabbit corneas decreases 87 to 100% of corneal epithelial ACh, suggesting a neuronal affiliation of this substance (126). Hemicholinium, when injected subconjunctivally or into the anterior chamber,

reduces ACh levels. When ACh levels fall by at least 40%, the cornea loses its touch sensitivity. The sensitivity returns concurrent with the return of ACh levels to normal values. It is postulated that ACh is a sensory mediator and may have a role in pain perception in the cornea.

In contrast to the studies of Fitzgerald and Cooper (126), Stevenson and Wilson (447) did not find changes in the rabbit corneal epithelial ACh and the loss of corneal reflex with hemicholinium. It is not possible to explain these differences. Some of the differences may be attributed to differences in the bioassay preparations for ACh or differences in anesthesia, or even collection of corneal epithelial samples and variability in the rabbits. It is possible that in the studies by Fitzgerald and Cooper (126), hemicholinium may have penetrated to the efferent side of corneal reflex resulting in blockade of synaptic transmission at the eyelid.

Trans-4(1-naphthylvinyl)pyridine (NVP), a choline acetyltransferase inhibitor, reduces corneal ACh by about 72% and does not abolish corneal reflex (447). In the absence of NVP, neostigmine increases ACh content in the cornea while in the presence of NVP, neostigmine does not increase ACh. It seems that ACh, which is subject to depletion by NVP, is also susceptible to hydrolysis by cholinesterase. Only 50% of the original ACh survives in the rabbit corneal explants cultured *in vitro* for 6 to 13 days (142). These studies suggest that there may be two pools of ACh in the rabbit cornea.

2. ACh in the Regulation of Corneal Water and Ion Transport. According to Williams and Cooper (487), the level of ACh appears to vary independently of the degree of hydration in bovine cornea. Further work is necessary before definite conclusions can be reached.

D. Silk Glands of Spiders

Although there are no detailed investigations on the influence of ACh and related agents on amino acid metabolism in experimental models, there are scattered observations that they influence protein synthesis. A stimulating action of the irreversible cholinesterase inhibitor, phospholine, on ^{14}C-lysine incorporation into proteins of the rat brain was described by Clouet and Waelsch (80). High doses of paraoxon (10^{-3} M) inhibited ^{14}C-acid incorporation into proteins (trichloroacetic acid precipitate) of the isolated nerve bundles from the walking legs of lobsters (478). Several cholinergic agents, physostigmine, carbachol, and paraoxon, have been shown to increase the incorporation of labeled alanine into fibroin, the protein in the silk glands of spiders (338). This stimulation by cholinergic agents is blocked by atropine. These effects of cholinergic agents can be divided into two components: effects on 1) uptake of amino acids by the cell or transport of amino acids across the cell membrane, and 2) actual incorporation of amino acids into proteins.

The silk glands of spiders, *Araneus diadematus* and related species, are discrete glands producing a single structural protein (fibroin) and therefore provide a good model for a pharmacologist to study drug effects on protein synthesis. The rate of incorporation of amino acids into the ampullate gland is stimulated by cholinergic agents both *in vivo* and *in vitro* (336, 337, 339). This stimulation is blocked by atropine. The ampullate glands can also be stimulated by emptying out the fibroin. This stimulation is not influenced by atropine. Thus there is evidence for the existence of two mechanisms to regulate protein production in the ampullate gland. In both cases, the first stage of the cycle of stimulation is the secretion of preformed protein droplets from the epithelium into the lumen. This is followed by accelerated synthesis of new protein. The two steps, secretion and synthesis, can be separated by pretreatment with a blocker of protein synthesis, such as puromycin or actinomycin D. The length of time for active synthesis and secretion varies with the mode of stimulation. If the stimulation is caused by emptying of the gland, this phase of the cycle lasts for about 8 hours; with cholin-

ergic stimulation, the duration is only about 4 hours. The difference is presumably due to the fact that the lumen of the gland remains full of protein in the case of cholinergic stimulation.

The importance of the cholinergic mechanism is not known. It is possible that it acts as a fine control in regulating the production of protein and is the means by which external stimuli can affect the amount of silk available for web spinning. Because ACh acts on the membrane cholinergic receptors, it may increase the uptake of amino acids. There may be a basal turnover rate of proteins in the gland, and this may be increased considerably by cholinergic stimulation. It is not known whether cholinergic stimulation increases amino acid uptake independent of protein synthesis. Cholinergic stimulation may increase the amino acid uptake and thereby protein synthesis in situations of greater demand. Spiders under the influence of physostigmine build larger webs.

E. Limb Regeneration in Salamanders

Regeneration of the amputated amphibian limb requires the presence of nerve (426). If the nerve is transected after growth has begun, the regenerate gradually breaks down in a distoproximal direction and is reabsorbed (53). Both sensory and motor fibers can initiate and maintain limb regeneration, although in the case of the sensory nerve, the trophic influence is opposite in direction to transmission of impulses from the periphery. Development of striated muscle depends upon release of ACh (100). Botulinum toxin, hemicholinium-3, and curare prevent development of striated muscle in the growing chick embryo. Fat replaces the muscle mass, a condition usually seen after long-term denervation.

Hemicholinium-3, which partially paralyzes the larval salamander profoundly, retards regeneration of the amputated hind limb (221). This drug also reduces the vascularity and mitotic index of the regenerating tissue. After withdrawal of the drug, the rate of regeneration returns to normal. Atropine (40 mg/kg) does not retard growth. These findings suggest that in the salamander, ACh may mediate neurotrophic activity.

Although there is much evidence supporting ACh as a neurotrophic factor (100), there are several reasons why it is not universally accepted. Infusion of ACh into a denervated limb does not restore its regenerative capacity. However, an infusion cannot duplicate neural activity. A more cogent reason for excluding ACh as the growth mediator is that the concentration of the substance in sensory nerves is negligible compared to that in motor nerves. Yet the sensory nerve is far more capable of influencing regeneration than is the motor component. On the other hand, regenerating tissue contains concentrations of ACh that are greater than normal but which then return toward normal during the period of early differentiation. These observations indicate that ACh may participate in other functions that may or may not fully relate to neuronal functions.

Prolonged treatment of the salamander with hemicholinium-3 or triethylcholine causes regressive and degenerative changes in taste organs and thinning of the lingual epithelium (218). Doubling the dose of hemicholinium-3 from 1.5 to 3 mg/kg does not appreciably increase the rate of taste bud degeneration. In the higher vertebrates, taste buds degenerate after surgical denervation (160, 161, 222, 360). The tongue epithelium atrophies after section of the glossopharyngeal nerve (162). In view of the fact that taste buds and lingual epithelium also degenerate after treatment with cholinolytic agents, the question arises whether these agents cause destructive nerve changes. However, serial sections of the tongue stained for nerve has revealed that nerve fibers are intact in the lingual epithelium despite drug treatment for 21 weeks. Studies using light microscopy indicate that atrophy of taste buds is apparently not caused by nerve fiber degeneration.

The lateral line organs are mechanore-

ceptors in the amphibian. Rapid degeneration of lateral line organs requires simultaneous destruction of lateral line nerve as well as the related spinal nerves (234). Although the lateral line organs regress significantly during treatment with hemicholinium-3, chronic administration of triethylcholine causes no detectable change in these structures despite substantial taste bud degeneration. Evidently, the lateral line organ is relatively resistant to the effects of this weaker cholinolytic agent, perhaps because of the dual innervation. The morphology of these nerves is unaffected by treatment with hemicholinium-3.

Treatment with hemicholinium-3 not only blocks synthesis of ACh but the drug also possesses an action like that of curare. While this may account for growth retardation of a mobile extremity, the taste buds and the lateral line organs are not likely to degenerate because of "disuse" produced by semiparalyzing doses of hemicholinium-3. Furthermore, triethylcholine produces persistant paresis, but lacks curariform activity (32), yet the drug retards limb regeneration (220) and also causes taste buds to degenerate. ACh may serve as a neurotrophic factor. This view is supported by the fact that treatment with hemicholinium-3 causes degeneration of taste buds and lateral line organs without causing histopathology of the nerve. However, this apparent neurotrophic action of ACh can be accounted for in other ways. First, ACh is known to alter the permeability of postsynaptic membranes, and thus may affect entry into the target tissue of a neurotrophic substance. According to Koelle (256), the release of ACh from the nerve terminals may cause an additional discharge of ACh; a trophic substance in the nerve may also be released in this way. Thus, ACh may be involved only indirectly in the trophic process.

Drachman and Singer (102), for example, failed to inhibit regeneration of the amputated limb of *Triturus viridescens* by the use of botulinum toxin, a compound that effectively blocks release of ACh. This indicates that regeneration is linked to the local synthesis of ACh but not to the release of preformed and stored ACh. Furthermore, taste buds of *Triturus* persist when transplanted to the liver of this amphibian (490), yet the liver has few nerves. Since the liver regenerates readily, even in man, the trophic factor presumably lies within the cell and not in nervous tissue, in keeping with the fact that embryonic or malignant tissue grows well without innervation. Information on the role of ACh in the regeneration of liver tissue, embryonic tissue, and malignant tissue is not available.

The skin of the gilled salamander contains large secretory cells rich in cholinesterase and innervated by unmyelinated fibers of fine caliber (219). During regeneration of the amputated limb, these Leydig cells have reappeared in the fresh skin. Treatment of the salamander with hemicholinium-3 or triethylcholine, but not atropine, prevents return of these cells and induces degeneration of the Leydig cells in the intact limbs. Denervation of the sacral plexus of intact hind limbs has also induced degeneration and disappearance of Leydig cells, indicating dependence on a neurotrophic factor. The activity of this factor may be influenced by ACh because treatment of the salamander with drugs that interfere with synthesis of ACh reproduces the effects of denervation. Investigations using specific choline acetyltransferase inhibitors are necessary to confirm the above results.

Leydig cells are mucus-secreting glands that, according to Kelly (243), may serve as ionic barriers in aqueous medium or provide fluid to the extracellular compartment of the epidermis when the animal is exposed to air. Regardless of their precise physiological significance, Leydig cells are evidently glandular tissue. Both Leydig cells and taste buds are apparently innervated by nerve fibers that are sensitive to cholinolytic drugs. In this respect, Leydig cells behave somewhat like taste buds. However, only occasional nerve fibers terminate on the Leydig cells despite rich subcutaneous

innervation. Yet both taste buds and Leydig cells are sensitive to denervation (220). All of these observations indicate that ACh does indeed mediate certain unidentified neurotrophic functions.

Narcotic analgesic compounds interfere with release of ACh in the peripheral and central nervous systems (439). Methadone and cyclazocine exhibit significant effects on the rate of limb regeneration in salamanders (217). The possible occurrence of endogenous opiate-like substances, enkephalins (216, 265, 439) and endorphins (144), in salamanders and their role in ACh release in this organism are not known.

Application of silastic cuffs containing either colchicine or vinblastine to sciatic nerves of rats produces electrophysiological signs of denervation (470). This effect has been attributed to the disruption of axonal flow and trophic factors by colchicine and vinblastine.

Trophic substances are not unique to neurons, but are also produced by all living cells. Apparently the neuron produces much more, and the excess quenches the production in target cells (428). Salamander limbs without the nerve (embryologically aneurogenic) do regenerate (456). When nerves are caused to grow into a fully developed aneurogenic limb, regeneration becomes nerve-dependent. This nerve dependency can be reversed on prolonged denervation (457). It will be interesting to determine the variations in the components of the cholinergic system during regeneration in aneurogenic limbs.

All of the above observations indicate that ACh has a non-neuronal function in limb regeneration that is not clearly understood at the present time. Several trophic systems besides that of the salamander were discussed in detail in recent symposia (101, 103).

V. Occurrence of One or More Components of the Cholinergic System in Cultured Cells and Developing Cell Systems

One or more components of the cholinergic system have been shown to occur in cells cultured in media or in cells of embryonic origin. Some of these cell types are developing neuronal cells. However, it has been shown that in these cells, ACh may have functions other than chemical transmission. Studies on limited cell types are summarized below.

A. Fibroblasts

L cells, a family of cell lines of mouse fibroblastic origin, generate a prolonged active membrane hyperpolarization (hyperpolarizing activation response) on mechanical or electrical stimulation (317). When applied iontophoretically, ACh elicits a similar response. Atropine blocks ACh but not the electrically or mechanically elicited responses. The importance of this response is not known but it may be the means by which the external stimuli communicate with cells. Some of these cell types contain ACh (361). If these cells can synthesize ACh and release it into the culture medium, released Ach in turn stimulates the cell to produce a hyperpolarization response. This may be the means by which the cell communicates with its external membrane and influences its functional properties.

The hyperpolarizing activation response can also be elicited by electrical, mechanical, or ACh stimulation of cells adjacent to the recorded cells. This response from one cell to another is not dependent upon direct electrical coupling between cells and is not blocked by application of a bath containing atropine or curare. Therefore, transmission of this response from cell to cell is humoral and noncholinergic.

There is a relationship between growth and acetylcholinesterase activity in these cells: 1) Although only low levels of acetylcholinesterase activity are present in low density exponentially growing cultures of L-929 cells, high density growth-inhibited cultures exhibit considerable enzyme activity approximately 100-fold greater than the basal level (140). 2) The activity of the enzyme invariably rises when cell division is arrested (350). 3) Clones with low saturation density have significantly higher basal and terminal acetylcholinesterase ac-

tivities than clones with high saturation density (425). These findings together with the occurrence of ACh in these cells and their responsiveness to ACh raise the interesting question of a possible relationship between components of the cholinergic system and cell membrane associated growth control mechanisms which need not be confined to neuronal cells (140).

B. Neuroblasts

The role of the cholinergic system in neuronal development has been reviewed (116, 123). It appears that all neurons are born with an ACh system marked on their birth certificates. In several embryonic nerve cells, acetylcholinesterase is found from the very earliest developmental stages. There is evidence for ACh synthesis in neuroblasts from the earliest stages (123).

The initial appearance of the cholinergic system is independent of the processes of synaptization. Each neuroblast population undergoes its own enzymatic development without necessarily being related to other populations. In skeletal and visceral regions, components of cholinergic system cannot be found before the penetration of neural elements. In skeletal and probably also in visceral muscle tissue, one component of the cholinergic system, acetylcholinesterase, is present early. However, the appearance of acetylcholinesterase is dependent upon the arrival of nerve fibers; an early release of ACh within myotomes initiates the synthesis of acetylcholinesterase within myoblasts (124).

There seem to be two different phases in the development of the cholinergic system of neuroblasts; the first phase may be considered as a primary, ubiquitous, and transient property of all differentiating neuroblasts (Table 2). The second phase develops exclusively in potential cholinergic neurons, and its appearance is critically related, at least chronologically, to the development of synaptic connections among different neuronal populations.

The physiological role of the first phase of the cholinergic system in neuroblasts is unknown. Its role may be related to transmembranal or transneuronal influences that represent the background of maturational growth of neuroblasts.

One possible transmembranal or transneuronal mechanism may involve the release of ACh by one neuroblast, which may affect the permeability of it own membrane or the membrane of other neuroblasts. Increase in membrane permeability may in turn facilitate membrane transport of nutrients and the synthesis of proteins by developing nerve cells (239) and phospholipids that are components of neuroblast membranes (287). The similarities between the patterns of proteins, RNA, and ACh synthesis in neuroblasts suggest a relationship between neural histogenesis and the cholinergic system.

The second phase of the developing cholinergic system is the basis of a well defined physiological event, namely the transmission of nervous impulses at cholinergic synapses.

Even in well developed cholinergic neurons, there are two components of ACh action, spontaneous release of ACh, which maintains miniature end place potentials (m.e.p.p.), and the bulk release of ACh upon nerve stimulation, which initiates the conducted impulse. The physiological function of the quantal release of ACh, other than its relationship to chemical transmission is not well understood. ACh may open pores in the postsynaptic membranes and increase the permeability to ions and energy metabolites. It may facilitate the uptake of tropic substances by muscle cells that are released from the nerve.

A question arises whether the first phase of the cholinergic system in developing neuroblasts is really transient or persists in some other form in the second phase. The function of ACh as a possible transmembranal transneuronal messenger in the first phase may be transformed as a messenger of cell-cell communication in the second phase, which is the basis of quantal release of ACh. The above question becomes more important in the light of hypotheses regarding (a) the role of ACh in the adrener-

TABLE 2

Properties of the first and second phases of the cholinergic system in developing nerve structures[a]

First Phase	Second Phase
1. Present in neuroblasts.	Present in cholinergic neurons
2. May be revealed very early, almost at the same time as neurofibrillary reaction and RNA perinuclear cap.	The well developed cholinergic neuron.
3. Independent of synaptic contacts.	Appears critically at the appearance of synapses, maintenance of synapses. Controls and regulates its activity.
4. Primitive property of neuroblasts (?)	Acquired property during development and is conditioned by extrinsic factors.
5. Primarily localized in cell bodies.	Spreads to neuronal processes.
6. Disappears with maturity or manifests itself in a different form (?)	Maintained for the life-span of the neuron.
7. Probably involved in the growth and maturation.	Involved in cholinergic transmission.
8. Formation of acetylcholine is not well understood.	Choline acetyl transferase involved in the formation of acetylcholine from choline and acetylcoenzyme A is well understood.
9. Sites of formation and action of acetylcholine are not known.	Sites of formation and action are well understood.
10. Components are not characterized.	All components are well characterized.

[a] Summarized from Filogamo and Marchisio (123).

gic neurons (50), (b) the role of ACh in the ACh-release amplification mechanism (256), (c) the role of ACh in non-nervous cells like human syncytiotrophoblast (396), and (d) the influence of ACh on protein synthesis in several types of cells. In the light of the specialized function for ACh in the second phase, the primary function of ACh in the first phase becomes secondary and possibly is masked.

C. Neuroblastoma Cells

Bromoacetylcholine, which was synthesized by Chiou and Sastry (73), inhibits effectively the growth of mouse neuroblastoma cells (C-1300) in cell culture (70). It also inhibits tumor growth in A/J mice and prolongs survival time. The selectivity of the inhibition of choline acetyltransferase by bromoacetylcholine in relation to its inhibition of other intracellular enzymes and its influence on the availability of precursors for ACh synthesis are not known. Further studies have yet to be conducted for establishing the antitumor activity of inhibitors of choline acetyltransferase. These studies are of considerable significance in view of the role of the cholinergic system in developing neuroblasts (see section V B) and the existence of the component of the cholinergic system in neuroblastoma cells (7, 12, 31, 408).

D. Hemopoietic Stem Cell

The cholinergic systems in erythrocytes and other blood cells and spleen are discussed in detail in sections V I and IV A2, respectively. The cholinergic system of the undifferentiated cells of bone marrow, such as CFU-S, are included in this section.

The pluripotent stem cells in the bone marrow of normal adult mice are either in a resting stage (G_0) or have a long cell cycle with only a small fraction occupied by DNA synthesis (53a). These spleen colony-forming (CFU-S) cells are insensitive to the cytocide action of ^{3}H-thymidine. These cells contain acetylcholinesterase. It is of interest to know whether these cells can synthesize ACh.

Carbamylcholine (10^{-14}–10^{-11} M) and ACh (10^{14}–10^{-11} M) in the presence of neostigmine (10^{-6} M) trigger CFU-S cells into DNA synthesis. *d*-Tubocurarine chloride (10^{-6} M) antagonizes this effect. These observations indicate that cholinergic mechanisms influence the proliferation of the hemopoietic stem cell. It is yet to be clari-

fied whether the cholinergic system of the circulating erythrocyte is the remains of a cholinergic mechanism associated with DNA synthesis in the undifferentiated cells of bone marrow, such as CFU-S.

VI. Cholinergic Systems in Blood Cells

It is necessary to establish that all components of the cholinergic system exist in each type of blood cell before its functional significance can be established. Considerable effort has been devoted to delineate the occurrence of ACh, choline acetyltransferase, and cholinesterases in the following blood cells.

A. Erythrocytes

1. Acetylcholinesterase. The capacity of erythrocytes to split ACh was described by Galehr and Plattner in 1927 (131). Subsequently, it has been established that the erythrocyte cholinesterase is specific for ACh and should be regarded as acetylcholinesterase (3).

The earliest evidence that increased acetylcholinesterase activity is characteristic of young erythrocytes was obtained by Pritchard in 1949 (353). When the erythrocytes of rats subjected to blood loss are separated by centrifugation into reticulocyte-rich and reticulocyte-poor populations, high acetylcholinesterase activity is found in reticulocyte-rich and low activity in reticulocyte-poor populations (353). Sabine (376, 377) applied the same procedures to blood from patients with various hematological disorders and obtained similar results. According to studies by Allison and Burn (4), the loss in acetylcholinesterase activity with time was compatible with an exponential decay curve, with the assumption that in normal subjects, cells of all ages are present in equal quantities. Subsequent studies have supported the theory that erythrocyte acetylcholinesterase activity is related to cell age except in patients with paroxysmal nocturnal hemoglobinuria.

A. LOCATION OF ENZYME ACTIVITY. Acetylcholinesterase is located on the cell membrane of the erythrocytes. After *in vitro* hemolysis, acetylcholinesterase activity can be recovered in the erythrocyte membrane (5, 35). Experiments with proteolytic enzymes, which do not traverse the erythrocyte membrane, have indicated that acetylcholinesterase or its active sites are located at or near the outer surface of human erythrocytes (20, 125, 199, 340). On the basis of a molecular weight of 90,000 or 180,000 per active site, about 0.2% of human erythrocyte membrane protein is acetylcholinesterase (19). There are two components in human erythrocyte acetylcholinesterase that can be separated by ion exchange chromatography (420, 421). There is genetic variation of these components (81).

B. SPECIES VARIATION. There is extensive species variation in the levels of erythrocyte acetylcholinesterase. Of all the mammals, man has the highest acetylcholinesterase activity and the cat has the lowest (193). It is about 50 times higher in man than in the cat. The erythrocyte acetylcholinesterase activities of chimpanzee and monkey are closer to that of man: about 71% and 64% of that in man, respectively (54). These activities in many laboratory animals (cat, rat, rabbit, dog, guinea pig) are about 2 to 14% of that in man.

There is an inverse distribution of acetylcholinesterase activity between erythrocytes and platelets in man and the cat (493). In man, the acetylcholinesterase activity is highest in the erythrocytes and almost absent in platelets, whereas, in the cat, acetylcholinesterase activity is very low in erythrocytes and extremely high in platelets. It would be interesting to know whether there is an inverse relationship between acetylcholinesterase activity of erythrocytes and plasma cholinesterase activity in different species. It may be that the total ACh-splitting activity per unit volume of total blood may be the same in all species.

C. VARIATION WITH MATURATION AND AGE. There seems to be an increase in acetylcholinesterase activity in erythrocytes with maturation and a decrease in this ac-

tivity with aging. Acetylcholinesterase activity of the newborn's circulating erythrocytes is considerably less than that of adults (233). Mean acetylcholinesterase activity of cord erythrocytes is 53% of that of adult erythrocytes and adult levels are reached by 3 to 5 months of age (45). During its life span of 120 days, the aging process of the erythrocyte is characterized by specific modifications. There is an increase in methemoglobin and specific gravity and a decrease in electric charge. It is possible to separate erythrocytes in accordance with their age by using techniques based on specific gravity (352). Acetylcholinesterase activity and activities of several intracellular enzymes decline as the erythrocytes age (37, 349).

The relationship between decline in acetylcholinesterase activity and structural and functional alterations that determine the ultimate removal of the aged erythrocytes from circulation are not understood. It remains to be elucidated whether the changes in acetylcholinesterase activity are related to an actual loss of enzyme protein or progressive enzyme denaturation or a modification of the enzyme with decreased catalytic efficiency.

A gradual decline in acetylcholinesterase activity can be observed with increasing age of erythrocytes in a normal adult (197). A more accelerated decrease in activity is seen in patients with reduced enzyme activity, either because of ABO hemolytic disease or because of autoimmune hemolytic anemia (197). An exception to the rule that acetylcholinesterase activity declines with advancing age of circulating erythrocytes is seen in paroxysmal nocturnal hemoglobinuria (9, 197). In this disease, this enzyme activity is higher in circulating older cells than in reticulocytes. This has been explained by the presence of a dual population of cells; one short-lived and another with normal survival time (235), one complement-sensitive and another complement-insensitive (269, 367). It is not clear whether incomplete maturation of these reticulocytes is responsible for their low acetylcholinesterase activity.

There are more reticulocytes (young erythrocytes) in the blood of neonates than in adults (330). Although acetylcholinesterase activity is increased in younger cells and decreased in older cells, the overall activity of this enzyme is higher in adults than neonates (45, 238). This dissociation between acetylcholinesterase activity and reticulocyte content is of special significance because such dissociation is not observed with other enzymes. Glucose-6-phosphate dehydrogenase (196) and inorganic pyrophosphatase (191), intracellular enzymes in erythrocytes, are increased in fractions containing the youngest cells and unseparated erythrocyte specimens from normal infants and from neonates with ABO disease (237). Therefore, the reduced acetylcholinesterase activity in ABO disease is not due to preferential elimination from the circulation of younger cells that possess A and B antigens, thereby leaving populations of older mean age (159).

Fetal erythropoiesis is greatly accelerated at the end of intrauterine life, and, therefore, a premature delivery of cells into circulation could take place (495). These premature cells could be similar to stress "reticulocytes" produced in the adult in response to hemorrhage or hemolysis (168, 362). During "stress reticulocytosis," the normoblasts skip the stage of orthochromatic normoblasts and lose their nuclei at the polychromatophilic stage to become reticulocytes. This incomplete maturation could be correlated with the dissociation of acetylcholinesterase activity and reticulocyte concentration in erythroblast infants as well as in all neonates of less than 3 months of life. In neonates of 3 months of life, acetylcholinesterase activity in red blood cells reaches adult levels (238).

D. RELATIONSHIP TO ION MIGRATION AND HEMOLYSIS. When human erythrocytes are suspended in isotonic sodium bicarbonate buffer, sodium enters and potassium leaves the cell and the cell hemolyzes, presumably

due to a net increase in base and water (157). When ACh is added to the suspension, the net rate of ion transfer decreases and hemolysis is delayed. Physostigmine in concentrations sufficient to inhibit acetylcholinesterase reverses the effect produced by ACh by approximately 85%. Benzoylcholine, which is not hydrolyzed appreciably by erythrocyte acetylcholinesterase, does not decrease the ion transfer or delay hemolysis when substituted for ACh (156–158, 208–210, 281). The order of activity of drugs in maintaining the integrity of the red cells is ACh > triacetin > acetyl-β-methylcholine > ethyl propionate > benzoylcholine. The ability of erythrocyte acetylcholinesterase to split these esters falls in the same order. When the ACh that is added to a suspension of erythrocytes is completely metabolized, migration of ions with the gradient occurs again (281). Not only physostigmine, but also other inhibitors of acetylcholinesterase, including methadone (157), diazonium salts (212), and neostigmine (210) produce permeability changes in erythrocytes.

These investigations indicate that the maintenance of the integrity of erythrocytes depends on the activity of the enzyme, acetylcholinesterase. When this enzyme is actively metabolizing ACh, the cell remains intact for a considerable period of time—probably as long as conditions for metabolism (*e.g.*, substrate concentration) remain optimal. When the enzyme is inactive either because of lack of the substrate or the presence of inhibitors, the cell loses its selective permeability and the cations migrate in the direction necessary to establish equilibrium with the environment. The mechanism whereby acetylcholinesterase maintains the integrity of erythrocytes is not known. Acetylcholinesterase is located on the cell membrane and a great decrease in free energy occurs when it hydrolyzes ACh. It has been suggested that this free energy is utilized to prevent the permeability changes in erythrocytes (208–210).

E. VARIATION OF ERYTHROCYTE ACETYLCHOLINESTERASE IN HUMAN DISEASE. Several enzymes have been identified in the erythrocyte membranes, but alterations in activity associated with human disease are found regularly only with acetylcholinesterase. This enzyme activity is reduced in paroxysmal nocturnal hemoglobinuria (PNH), an acquired hemolytic disorder in which a certain proportion of erythrocytes is abnormally sensitive to complement and complement-fixing antibodies (9, 105, 292), and in ABO hemolytic disease of the new born (ABO-HDN), a transient disorder of the neonatal period characterized by accelerated antibody-mediated erythrocyte destruction in blood group A or B infants of blood-group O mothers (196, 237, 432, 448). Severe poisoning with specific anticholinesterase agents reduces erythrocyte acetylcholinesterase activity (143). Subnormal levels of this enzyme in erythrocytes are found occasionally in some individuals with autoimmune hemolytic anemia (AIHA), a disease in which the red cells are susceptible to destruction by circulating antibodies with affinity toward the subject's own cells (196, 416, 430, 455). Severe poisoning with specific anticholinesterase agents reduces erythrocyte acetylcholinesterase activity (215), anticholinesterase activity is also diminished in the presence of acute leukemia (193), but the significance of this is not understood.

There is no information on the mechanisms responsible for reduction in erythrocyte acetylcholinesterase activities in paroxysmal nocturnal hemoglobinuria, ABO hemolytic disease of the neonate, or autoimmune hemolytic anemia. There is no evidence indicating that reduction in acetylcholinesterase activity is related to progressive enzyme denaturation, a progressive loss of enzyme protein, or altered enzyme characterized by decreased catalytic efficiency. Although the physiological functions of this enzyme in the erythrocytes have not been elucidated, its occurrence near the outer cell surface (21, 302) gives it special significance in studies of cellular

TABLE 3

Substances that inactivate acetylcholinesterase in erythrocytes and their effects on the properties of erythrocytes[a]

Properties of Erythrocytes	Proteases	Sulfhydryl Compounds[b]	Cephalothin (Antibiotic)	Fluorinated Nitrobenzenes[c]	Tannic Acid	Aldehydes[d]
1. Inactivate acetylcholinesterase	Yes	Yes	Yes	Yes	Yes	Yes
2. Mimic paroxysmal nocturnal hemoglobinuria cells	Yes	Yes	Yes	No	No	No
3. Alter permeability	Yes	Yes	?	Yes	Yes	Yes
4. Reduce *in vivo* survival	Yes	Yes	No	?	?	?
5. Decrease osmotic resistance	Yes	Yes	Yes	?	Yes	?
6. Release mucoids from cell surface	Yes	?	?	?	?	No
7. Alter electrophoretic mobility	Yes	?	?	?	?	Yes
8. Affect cell agglutinability	Yes	No	No	Yes	Yes	Yes
References:	199	236, 433	122, 198, 295, 431	21, 129, 195	192, 202, 354	193, 194

[a] General reviews (193, 194).

[b] Cysteine, glutathione, penicillamine, 2-aminoethylisothiouronium bromide.

[c] 1-Fluoro-2,4-dinitrobenzene; 1,5-difluoro-2,4-dinitrobenzene.

[d] Formaldehyde, glutaraldehyde.

membranes, and specific acetylcholinesterase alterations seen in several hemolytic disorders may be of importance in understanding certain basic disease processes at the cellular level.

In order to study the nature and significance of the reduced erythrocyte acetylcholinesterase levels, several investigators have tried to develop methods for *in vitro* reproduction of the enzyme defects. These methods are concerned with the action of exogenous substances on erythrocytes with special emphasis on the production of acetylcholinesterase deficient erythrocytes. All of these methods are summarized in Table 3. For details, reviews by (Herz and Kaplan (193, 194) may be consulted. Although erythrocytes that are deficient in this enzyme are produced *in vitro* by a variety of chemically unrelated substances that affect the erythrocyte membrane in different ways, the questions of the role of acetylcholinesterase in erythrocytes and the relationship of reduced levels of this enzyme to hemolysis remain unsettled.

2. Choline Acetyltransferase. Acetone-dried powder of human erythrocytes synthesizes ACh (6–50 μg/g dried powder/hr) when it is incubated with choline, coenzyme A, and ATP (211, 289).

3. Acetylcholine. The content of ACh, determined by bioassay, in human erythrocytes is about 0.08 μg/ml. This low level of ACh indicates that erythrocytes do not contain any storage sites for ACh, and the ACh turnover rate may be higher than in nervous tissue.

4. Muscarinic Receptors. Recent direct binding studies have indicated that there are muscarinic receptors in erythrocyte membrane (8a). However, further work is necessary to establish unequivocally the existence and function of muscarinic receptors in erythrocyte membrane.

B. Platelets

Platelets from several species have been shown to contain acetylcholinesterase and choline acetyltransferase.

1. Acetylcholinesterase. The cholinesterase in human platelets and several other species is acetylcholinesterase (76). It is specific for splitting ACh and acetyl-β-

methylcholine. High concentrations of ACh cause substrate-inhibition of this enzyme. As in erythrocytes, this enzyme is associated with cell ghosts of platelets and is therefore membrane bound (494). This enzyme activity is probably located within the platelet, rather than on the platelet, and is loosely bound. Part of the enzyme activity can be recovered in the soluble fraction after subcellular fractionation. The actual location of this enzyme activity is within the cell and the mechanism whereby this enzyme appears in the soluble fraction is not known.

2. Release of 5-Hydroxytryptamine, Adenine Nucleotides, and Acetylcholinesterase from Platelets. Acetylcholine, acetyl-β methylcholine, and thrombin release acetylcholinesterase, 5-hydroxytryptamine, and adenine nucleotides simultaneously from canine platelets. The parallel release indicates that they may come from the same intracellular compartment (77). ACh also causes aggregation of canine platelets (375). Due to the observations that acetylcholinesterase may play a role in the aggregation of canine platelets, this effect seems to be specific for canine platelets.

Acetylcholine and acetyl-β-methylcholine do not induce aggregation of human platelets (423). They do not release adenine nucleotides and acetylcholinesterase from human platelets (77). The release of adenine nucleotides and acetylcholinesterase, as well as the occurrence of aggregation, takes place with human platelets only when thrombin is used as an inducing agent (77). The thrombin-induced release of adenine nucleotides is comparable in canine (50%) and human platelets (49%), but the release of acetylcholinesterase in canine platelets (31%) is much higher than in human platelets (13%). However, the acetylcholinesterase in canine platelets is about 103 times higher than in human platelets. The ACh-induced release reaction as well as the aggregation reaction in canine platelets is blocked by atropine. All of these observations indicate a role for acetylcholinesterase in canine platelets. The role of acetylcholinesterase in human platelets has yet to be established.

3. Choline Acetyltransferase. Choline acetyltransferase activity has been shown to be present in canine and human platelets (4×10^{-6} μmol ACh synthesized per 10^8 platelets). Both canine and human platelets have the same capacity for the synthesis of ACh; but the canine platelets have about 103 times more acetylcholinesterase. Therefore, the turnover rate of ACh in human platelets should be considerably higher than that in the canine platelets.

Choline acetyltransferase of canine platelets is a cytoplasmic enzyme, since no activity can be detected with intact cells, and most of the activity (70% or more) is retained in the soluble fraction after platelets are lysed and centrifuged at $100{,}000 \times g$ for 1 hour.

Choline acetyltransferase activities are reduced when platelets are aggregated in response to thrombin regardless of whether platelets are suspended in buffer or in plasma. This reduction in choline acetyltransferase is greater in plasma (50%) than in a buffer (10%) at pH 7.4. The reasons for this reduction in choline acetyltransferase activity are not known.

4. Acetylcholine and Acetylcholine-Receptor. Although platelets have the capacity for the synthesis of ACh, there is no evidence that there is any mechanism for the storage of ACh in platelets. Although ACh induced release of adenine nucleotides and serotonin from canine platelets, and aggregation of platelets is blocked by atropine, there is no evidence from binding studies for the presence of muscarinic receptor in these platelets.

C. Lymphocytes

Studies on the components of the cholinergic system in lymphocytes are limited in number. An operational cholinergic system and a cholinergic receptor are indicated by indirect approaches—namely, augmentation of the cytotoxic response of thymus-derived lymphocytes, increases in cyclic GMP levels, increases in protein and RNA

synthesis, and increased lymphocyte motility in the presence of cholinergic agonists (163, 223, 452, 463). In all these situations, the cholinergic response is decreased or blocked by atropine, a specific muscarinic receptor blocking agent. Therefore, the response to ACh in the above situations is mediated through a cholinergic receptor of muscarinic type. This was recently confirmed by binding studies using (^{3}H) quinuclidinyl benzilate, a specific cholinergic muscarinic ligand. Each lymphocyte contains approximately 200 muscarinic receptors. Quinuclidinyl benzilate has a dissociation constant of 10^{-9} at these receptors and its binding to lymphocytes is blocked by atropine (147).

D. Leucocytes

There are no studies on the nature of the components of the cholinergic system in leucocytes. According to preliminary studies of Granitsas (152), ACh (50 μg/ml) and carbamylcholine (2.5 μg/ml) increased the uptake of labeled amino acids (^{14}C-alanine and ^{14}C-leucine) into proteins of the rat leucocytes *in vitro*. These effects of ACh and carbamylcholine are not influenced by neostigmine and atropine. Further work is necessary to evaluate the selectivity of these effects. Whether these effects are due simply to an increased transport or direct stimulation of protein synthesis or both is not clear.

VII. Cholinergic System in Spermatozoa

Szent-Gyorgyi (453) developed the glycerine fiber muscle model as a standard experimental system in the field of the pharmacology of muscle. Subsequently, this model has been used by a number of investigators to study nonmuscular systems (25, 205). Nelson (315) has reviewed the evidence that contractile proteins are responsible for movement in a variety of cells and organelles, including spermatozoa. They are described as actin-like, myosin-like, and actinomysin-like proteins that display many of the biochemical and physiological parameters of contractile proteins of the muscle. The motility of spermatozoa is generally compared with the automaticity of smooth muscle, and it has been suggested that an ACh-cycle might be responsible for the contraction and relaxation cycles of spermatozoa.

Although detailed characterization of the various components of the spermatozoal cholinergic system are lacking, it has been demonstrated that ACh, choline acetyltransferase, and acetylcholinesterase occur in mammalian spermatozoa (27, 28). Several pharmacological studies have indicated that there are cholinergic receptors of a nicotinic type in spermatozoa. Complete characterization of the various components of spermic cholinergic system is necessary before the functional role of this system in spermatozoa can be evaluated.

A. Acetylcholine

1. Occurrence. The occurrence of ACh-like substances in the cytoplasm of rabbit, bull, ram, and boar spermatozoa was demonstrated by Saiko (378), using bioassay techniques. More recently, Bishop *et al.* (27, 28) have collected spermatozoa from fresh ejaculates of bull and man, washed them, and extracted them with acetonitrile. The purified ammonium compounds from the acetonitrile-extracts were subjected to pyrolysis gas chromatography. In the gas chromatogram of the quaternary ammonium compounds of bull spermatozoa, two peaks were found and identified as 2-dimethylaminoethyl acetate and 2-dimethylaminoethyl propionate. In the gas chromatogram of the quaternary ammonium compounds of the human spermatozoa, a peak for 2-dimethylaminoethyl acetate was found. These peaks disappeared when the extracts were subjected to hydrolysis by acetylcholinesterase or alkali, and the peak heights were enhanced when the corresponding exogenous choline esters were added to the extracts. These observations suggest that ACh and propionylcholine occur in bull spermatozoa and that ACh occurs in human spermatozoa. There are cer-

tain limitations in the identification of peaks in gas chromatography by retention times because they are influenced by impurities. Therefore, combined pyrolysis gas chromatography and mass fragmentography (GC/MF) was also used for the identification of choline esters in bull spermatozoa (29). In this procedure, choline esters are demethylated and the demethylated esters are assayed using GC/MF by focusing on *m/e* 58, which is the most prominent fragment. This fragment corresponds to dimethylenimmonium ion $(CH_3)_2\overset{+}{N}{=}CH_2$. These studies have demonstrated the occurrence of ACh in human spermatozoa, and ACh and propionycholine in bull spermatozoa. According to the estimates, bull spermatozoa contains 4.3 ± 1.4 pmol (mean ± S.E.) of ACh/10^6 cells and 0.2 ± 0.1 pmol of PCh/10^6 cells, and human spermatozoa contains 28.6 ± 3.4 pmol of ACh/10^6 cells. Spermatozoa also contain other ammonium compounds that have not been identified.

The occurrence of propionylcholine in bull spermatozoa is not surprising because both choline esters, ACh and propionylcholine, are known to occur in spleens of oxen and sheep (14, 132, 190). Propionyl-coenzyme A is known to occur in ruminants (242) and can be synthesized from propionyl-coenzyme A and choline in the presence of choline acetyltransferase (347). It remains to be seen whether propionylcholine can be detected in spermatozoa of other ruminants.

2. Nature of Occurrence. The occurrence of ACh in membrane stores in nervous tissue is well established. In order to analyze for membrane stores of ACh in the sperm, fresh washed bull spermatozoa were divided into two fractions by Bishop *et al.* (28). One fraction was refrigerated at 4°C for 2 days and the other fraction was frozen for 2 days at −12°C. If ACh is separated from acetylcholinesterase by a membrane, then the ACh should be preserved in the sperm extract stored at 4°C. Freezing disrupts the membranes due to the formation of ice crystals. If the frozen sperm is thawed, ACh will be hydrolyzed by acetylcholinesterase. ACh was not present in either refrigerated or frozen sperm. These experiments indicate that ACh does not exist in a stored form within the membranes of the sperm. Compared to nerve terminals and human trophoblast (section VIII A3c) in which ACh occurs in a stored form, ACh occurs in spermatozoa in very small quantities. It seems that ACh is utilized in spermatozoa as it is synthesized.

B. Choline Acetyltransferase

Spermatozoa from the bull and man contain choline acetyltransferase. Bull sperm has greater choline acetyltransferase activity than human sperm. The choline acetyltransferase activities of the bull and human sperm are about 200 and 130 pmol of ACh formed/10^6 cells/10 min, respectively (26–29). It is yet to be determined whether this ACh is synthesized by an enzyme similar to that in the nervous tissue or by a different enzyme.

C. Cholinesterases

A cholinesterase-like enzyme has been demonstrated in spermatozoa of several mammalian and marine invertebrate species (303, 309, 419, 458). The enzyme of the pig and bull was identified as acetylcholinesterase using specific substrates and inhibitors. The enzyme selectively hydrolyzes ACh but is less effective in hydrolyzing butyrylcholine and benzoylcholine. The enzymatic hydrolysis of ACh shows an optimum substrate concentration of about 1.4×10^{-2} M. Physostigmine is a competitive inhibitor of bull sperm acetylcholinesterase. Ethylmercuric thiosalicylate (Merzonine), which inhibits horse serum cholinesterase, does not inhibit pig sperm acetylcholinesterase.

D. Choline Acetyltransferase and Acetylcholinesterase Activities in the Sperm from Various Segments of Epididymis

The various segments of epididymis (caput, proximal corpus, distal corpus, proxi-

mal cauda, and distal cauda) have been analyzed for choline acetyltransferase activity (169, 171, 172, 175) in the rabbit and the rat. Lowest activity was found in the caput, with increasing levels in the proximal and distal corpus and proximal cauda. Highest levels were found in the distal cauda. Although the role of the enzyme in epididymis is not completely understood, these observations indicate that in fully matured sperm, high levels of choline acetyltransferase are developed. In view of high concentrations of the above two enzymes in the tails of spermatozoa, these observations indicate that the spermatozoal cholinergic system is involved in their progressive motility (section VII E).

E. Choline Acetyltransferase and Acetylcholinesterase in Sperm Fractions

Choline acetyltransferase and acetylcholinesterase activities in the three sperm fractions (head, midpiece, and tail) can be determined by subjecting a suspension of washed bull spermatozoa to 20 kc of ultrasonic vibration and separating the fractions by centrifugation (28, 303). A 90 to 95% pure preparation can be obtained with this method. All fractions contain significant choline acetyltransferase and acetylcholinesterase activities. Specific activities of both these enzymes in the tail fraction are about 5 times higher than the corresponding values for the head or the midpiece (26, 28, 303, 309).

The cholinergic receptors in spermatozoa are considered to be of the nicotinic type (section VI D). The degree of binding of ^{125}I-α-bungarotoxin to tails of bull spermatozoa is about twice that for heads (26, 29, 383). Although the specificity of binding is not known, there must be some nonspecific binding as indicated by the relative distributions of choline acetyltransferase, acetylcholinesterase and α-bungarotoxin binding proteins in bull spermatozoa.

Information about the distribution of choline acetyltransferase in sperm fractions of species other than the bull is not available. The distribution of acetylcholinesterase in the ram, trout, and perch spermatozoa has been determined (see 28). Acetylcholinesterase in ram spermatozoa was largely confined to the tail fragments (28), while it occurred mostly in the heads of trout and perch spermatozoa (458). There are significant differences in the distributions of choline acetyltransferase and acetylcholinesterase in bull and marine organisms.

F. Effects of Cholinergic and Cholinergic Blocking Agents on Sperm Motility

1. Sea Urchin Sperm. If a cholinergic system plays a significant role in sperm motility, there should be a cholinergic receptor in the sperm cell membrane. Such cholinergic receptor must be responsive to cholinergic agonists and antagonists, and corresponding changes should be observed in motility. A number of cholinergic agents and cholinergic blocking agents have been used by Nelson (305, 307, 310–313) to characterize the cholinergic receptors in sperm from sea urchin, *Arbacia punctulata* (Table 4). Most of the agents exerted biphasic effects, stimulation of motility in micromolar amounts and inhibition of motility in millimolar concentrations. These agents include cholinomimetics (ACh, nicotine), muscarinic receptor blocking agents (atropine), and nicotinic receptor blocking agents (nicotine in high concentrations, *d*-tubocurarine, decamethonium, α-bungarotoxin). Of these agents, the classic nicotinic agonist, nicotine, and the classic nicotinic receptor blocking agent, α-bungarotoxin, are most effective in influencing the motility of the sea urchin sperm. On this basis, the receptor in sea urchin sperm can be classified as a nicotinic receptor of type 1, which is similar to the nicotinic receptor on the skeletal muscle. On this receptor, *d*-tubocurarine and atropine seem to be equally effective in causing depression of motility. There are no reported studies on the effects of specific choline acetyltransferase inhibitors and calcium antagonists on the motility of sea urchin sperm.

Several aspects of sperm motility—con-

TABLE 4

Effects of various cholinergic and cholinergic blocking agents on the motility of sperm from the sea urchin[a]

Pharmacological Agent	ED50 for Motility Depression	Concentration for Maximal Stimulation (% Control)[b]	Concentration for Maximal Inhibition (% Control)[b]	Effective Concentration Range
1. Cholinomimetics				
Acetylcholine	10^{-2} (15%)	10^{-5} (120)	10^{-2} (85)	10^{-5}–10^{-2}
Acetylcholine + DMSO[c]	10^{-2} (7%)	10^{-6} (175)	10^{-2} (107)	10^{-5}–10^{-2}
Nicotine	10^{-6}	10^{-8} (175)	10^{-3}	10^{-13}–10^{-3}
2. Anticholinesterases				
Physostigmine	10^{-3} (−40%)	10^{-6} (130)	10^{-3} (60)	10^{-7}–10^{-3}
Neostigmine	10^{-3} (−5%)	10^{-4} (110)	10^{-3} (95)	5×10^{-5}–10^{-3}
Neostigmine + DMSO	7×10^{-4}	5×10^{-7} (120)	10^{-3} (40)	10^{-11}–5×10^{-3}
3. Muscarinic receptor blockers				
Atropine	10^{-4}	5×10^{-9} (155)	10^{-3} (25)	10^{-10}–10^{-3}
4. Nicotinic receptor-1 blockers[d]				
d-Tubocurarine	7×10^{-3} (−20%)	5×10^{-4} (120)	7×10^{-3} (80)	10^{-4}–7×10^{-3}
d-Tubocurarine + DMSO	10^{-3} (−30%)	5×10^{-5} (85)	10^{-3} (70)	5×10^{-5}–10^{-3}
Decamethonium	9×10^{-5}	10^{-5} (120)	10^{-3} (10)	5×10^{-7}–10^{-3}
Succinylcholine + DMSO	5×10^{-3} (−15%)	5×10^{-7} (175)	5×10^{-3} (85)	5×10^{-9}–5×10^{-3}
α-Bungarotoxin	5×10^{-7}	—[e]	10^{-6}	10^{-7}–10^{-5}

[a] All concentrations are expressed as moles/liter; summarized from Nelson (304,307).

[b] Wherever indicated in parentheses.

[c] DMSO, dimethylsulfoxide; it increases the permeability of quaternary ammonium compounds into spermatozoa.

[d] Nicotinic receptors are of two types: N-1 at the somatic neuromuscular junction and N-2 ganglia. The effects of N-2 blocking agents (*e.g.*, mecamylamine) on sperm are not known.

[e] No stimulation was observed.

tractile system, cytochemical aspects, and flagellar movement and its regulation—have been studied and reviewed by Nelson and his collaborators (306, 308, 314, 316). In the present review, special attention is devoted to the components of the cholinergic system only.

2. Human Sperm. There are limited numbers of studies on the effect of cholinergic and cholinergic blocking drugs on mammalian sperm motility. In view of the reported species variations in the distribution of the components of cholinergic systems in sperm from mammalian and marine species, such studies will be of considerable significance.

According to Sanyal and Khanna (380), ACh produces opposite effects in two different types of human spermatozoa, which can be identified under microscopic viewing, one with a round head and the other with an oval head. The number of cells crossing a microscopic field in a given time period can be used as the index of motility. In the absence of drug, the number of the two types of cells crossing the microscopic field per minute are equal. In the presence of ACh, the number of round cells crossing the view decreases while that of the oval cells increases; the total number of cells crossing does not change. The motility of the oval cell increases as the concentration of ACh increases, reaching a maximum at 1/ml, and then declines rapidly to a lower level that is still higher than the control. The motility of the round cell remains subnormal throughout the range of ACh concentrations. The effect of ACh is poten-

tiated by physostigmine and is blocked by atropine.

The lack of membrane stores for ACh in spermatozoa indicates that ACh synthesis, the stimulation of the receptor by ACh, and the hydrolysis of ACh by acetylcholinesterase are closely linked and may be localized within the same compartment. In view of this unique organization of the cholinergic system in spermatozoa, inhibitors of choline acetyltransferase should exhibit dramatic effects on sperm motility. Recently, choline acetyltransferase inhibitors that are suitable for these studies have become available (64, 368). One of these potent inhibitors is 2-benzoylethyltrimethylammonium (BETA), which inhibits choline acetyltransferase from monkey brain (IC_{50}, 4.8×10^{-6} M), human placenta (IC_{50}, 1×10^{-6} M), and rat spermatozoa (IC_{50}, 8.5×10^{-8} M) at concentrations higher than 10^{-8} M after a contact time of 5 to 60 minutes (384). It depresses the motility index by about 80% after 5 minutes and by 95% after 1 hour at a concentration of 10^{-6} M. These observations indicate that the spermic cholinergic system controls the motility of sperm cells.

G. Sterility and Spermatozoal Cholinergic System

At the present time, sterility can be produced by affecting spermatozoa in the epididymis, so that: 1) they become immotile (*e.g.*, cyproterone acetate); 2) they are able to move in the vicinity of eggs but cannot fertilize them (*e.g.*, α-chlorohydrin); and 3) they fertilize eggs, but the embryos cannot develop beyond early stages (*e.g.*, methyl methane sulfonate, MMS; trimethylphosphate, TMP) (226).

Among the above compounds, trimethylphosphate (750 mg/kg, intraperitoneally) lowers choline acetyltransferase activity of sperm in all segments of epididymis and produces reversible sterility in both rats and rabbits (169, 171, 172, 176). The above dose of trimethylphosphate does not bring about major changes in brain choline acetyltransferase levels. Trimethylphosphate is a poor inhibitor of choline acetyltransferase *in vitro* (26, 169). These observations indicate that trimethylphosphate may interfere with maturation of spermatozoa in the epididymis. It is not known whether trimethylphosphate depresses the development of other enzymes and therefore the overall maturation of epididymal spermatozoa.

The problem of controlling fertility in the male by pharmacological means has not received much attention compared to the volume of research devoted to the female. Although progress in research has been slow in this area, several compounds are now available for inhibiting sperm production or function in experimental animals (224–227, 318).

VIII. Cholinergic Systems in Placenta

Human placenta has been investigated more extensively than animal placenta for components of the cholinergic system. It is now well established that an active cholinergic system exists in human placenta but its function is still unknown.

A. Human Placenta

Among the four components of the cholinergic system, there is good evidence for the presence of ACh and choline acetyltransferase in high concentrations in human placenta. There are low levels of acetylcholinesterase in placenta of gestation period 36 to 42 weeks. In view of the vascularity of placenta and the high concentration of cholinesterases in the maternal and fetal bloods, there is no definite physiological requirement for high concentrations of acetylcholinesterase in human placenta. There is some evidence for the presence of a cholinergic receptor of muscarinic type in the human placenta. This receptor may differ from the classic muscarinic receptor in some of its properties. Existence of such a receptor has yet to be confirmed by binding studies using agonists and antagonists. The physiological links between the various components of the human placental cholinergic system have yet to be investigated.

1. Cholinesterases in Human Placenta. The presence of cholinesterases in human placenta has been indicated by a number of investigations (174, 264, 329, 460, 492). The human placental enzyme has been characterized as acetylcholinesterase by Ord and Thompson (329) and by Koshakji *et al.* (264). Acetyl-β-methylcholine is a specific substrate for the placental acetylcholinesterase, which does not hydrolyze benzoylcholine to a significant degree.

According to studies of Harbison *et al.* (174) there is variation in the occurrence of acetylcholinesterase in human placentae. High concentrations of acetylcholinesterase were found in the placentae of gestation period of 16 to 22 weeks, using a sensitive radiometric method. These concentrations of acetylcholinesterase are comparatively lower than those in the nervous tissue or erythrocytes.

Recent studies raised questions regarding the occurrence of acetylcholinesterase in term placentae (374). Part of the acetylcholinesterase activity in human term placentae may represent contamination from maternal erythrocytes. No acetylcholinesterase could be detected in histochemical studies using the copper thiocholine method. In these sections, trapped intact erythrocytes and the stroma of hemolyzed red cells were found. The contamination from maternal erythrocytes to term placentae acetylcholinesterase can be explained more easily than the variation in acetylcholinesterase levels with gestation period. These studies do not exclude the possibility that acetylcholinesterase occurs in human placenta at other gestation periods.

Approximately 50% of human term placenta is blood (249). Therefore, there are adequate cholinesterases for the hydrolysis of ACh, if it is released into the maternal or fetal circulations. In the placenta, ACh seems to be in a bound form and is not available for hydrolysis by cholinesterases (section VIII A3). Therefore, occurrence of acetylcholinesterase in the human placenta itself is not crucial. Several other aspects of acetylcholinesterase are discussed with ACh in appropriate sections for convenience.

2. Choline Acetyltransferases in Human Placenta. It was shown by Comline in 1946 that there is choline acetyltransferase in human placenta (84). The placental homogenates of several other species—guinea pig, dog, cat, mouse, horse, and cow—have been shown to synthesize ACh in the presence of exogenous acetylcoenzyme A (471). These homogenates synthesized other products besides ACh that were not identified. A number of investigators have obtained partially purified preparations of choline acetyltransferase from human placenta (42, 296, 366, 390, 410). Recently, it has been shown that brain choline acetyltransferase and placental choline acetyltransferase will synthesize ACh by the same enzyme mechanism, namely the Theorell-Chance mechanism (390, 393). The effects of several choline acetyltransferase inhibitors on both human placental choline acetyltransferase and typical choline acetyltransferases prepared from neuronal sources are similar.

High concentrations of choline acetyltransferase are in the human placental villi, possibly localized in the syncytiotrophoblast layer, which separates the maternal circulation from the fetal circulation (396). Several other aspects of human placental choline acetyltransferase are discussed in the section on ACh for convenience.

3. Acetylcholine. In the early studies, ACh-like activity in placenta was detected using different bioassay methods. In several cases, ACh was not definitely identified. These studies are discussed separately from those in which ACh was definitely identified and measured by chemical methods.

A. ACh-LIKE ACTIVITY IN PLACENTA. Several authors have investigated the occurrence of ACh in placenta. The ACh content in all these studies was determined using bioassay preparations. Several naturally occurring substances behave like ACh, and placenta contains several biogenic amines. For convenience, these studies are discussed separately. If other ACh-like compounds are to be discovered, these studies

may also apply for them. The results of these studies are contradictory or divergent.

Bischoff *et al.* (24) demonstrated the presence of ACh-like activity in the human placenta in significant quantities. They measured ACh-like activity in the frog heart. They also investigated the uterus and found no ACh-like substances in human uterus. According to them, placental ACh-like substances act in the uterus and initiate contractions.

According to Haupstein (178), there exists in the human placenta ACh-like activity, 2 to 8 times higher than that observed by Bischoff *et al.* (24). He also stated that the amount of ACh-like substances in the placenta before term is certainly higher than after normal delivery. After having studied the different factors, which could modify the local conditions, he did not attribute to ACh-like activity of the placenta the role of triggering the contractions.

The studies by Chang and Gaddum (60) confirmed that the human placenta contains large quantities of a substance which, with the aid of several pharmacological and chemical tests, could be identified as acetylcholine, but the values obtained were much lower than those published previously, explained by the superiority of their bioassay methods, which has been confirmed. Chang and Gaddum (60) did not attribute any kind of physiological role to the large ACh-like activities found.

According to additional studies of Chang *et al.* (62) and Wong and Chang (489), the content of ACh-like activity in the placenta remains almost constant until delivery, at which time it falls greatly. This observation supports the hypothesis that ACh-like activity intervenes in the start of delivery. ACh-like compounds seem to be localized in the placenta at the level of "cotyledons." The fetal membranes, the cord, and amniotic fluid do not contain any trace of it.

The acetylcholine-like activity of the placenta is present in two forms (59): a free form and a bound form which could constitute a store. These facts have been confirmed by experiments involving perfusion of the placenta in saline solutions at 37°C.

According to Strack *et al.* (451), at the time of the birth of the child, the human placenta contains only very small quantities of acetylcholine-like activity. This amount then increases rapidly, becoming higher in proportion to the length of time during which the placenta remains in the uterus. A case was cited in which the placenta 1¾ hours in the uterus after delivery of the child contained ACh-like activity of 1512 nmol/g of wet tissue. After the rupture of the amniotic sac and particularly after the birth of the child, there occur important changes in the uterus. According to these investigations, the notable increase of ACh-like activity may be related to uterine contractions and mechanisms associated with birth. The proportion of ACh-like activity increased greatly with death of the fetus. It appeared then that ACh-like activity could serve as a hormone generated at the site of its action.

The extraordinarily high concentrations of ACh-like activity reported by Bischoff *et al.* (24), Haupstein (178), and Strack *et al.* (451), were contested by Chang and Gaddum (60). But, according to Heirman (185), the human placenta contains ACh-like activity only up to about 980 nmol/g. This activity is highest in membranes, medium in the cotyledons, and minimal in the cord. The content of ACh-like activity rises in the placenta between 3 and 6 months of pregnancy and remains almost steady up to and immediately after childbirth. A marked decrease in ACh-like activity then occurs within 35 minutes. The fall in ACh-like activity does not occur exactly with expulsion of the child, but rather during the period between expulsion of the child and expulsion of the placenta (185); 68% of the total ACh-like activity in placenta disappeared during the first 35 minutes in which the placenta remains within the uterus after the expulsion of the child. It appears that it is not the contractions of the uterus during delivery, but the functional modifications in the placenta that occur through the

binding of the cord (ligature) after childbirth, that bring about the decrease in ACh-like activity. This consideration makes it improbable that ACh-like activity intervenes in starting uterine contractions during labor, as had been suggested previously.

B. IDENTIFICATION OF ACh IN HUMAN PLACENTA. As we have already discussed, several investigators have reported the occurrence of ACh-like activity, determined by bioassays, in human placenta. The ACh-like activity may represent several substances. In recent investigations by Sastry *et al.* (395–397), the major component of this ACh-like activity has been identified as ACh by separation, using ion-exchange chromatography and gas chromatography. According to data obtained from 34 placentae, human term placenta contains 112 ± 7 nmol of ACh/g of wet tissue. The same group of placentae contained an unidentified compound X in small amounts (16 ± 2 nmol of ACh equivalents/g of wet tissue). This compound was detected in 97 to 98% of the placentae analyzed.

Since, according to Chang (59), the ACh-like activity of term placental extracts is equivalent to about 180 to 230 nmol of ACh/g of wet tissue and Sastry *et al.* (395–397) found about 112 nmol of ACh/g of wet tissue, about 55% of the ACh-like activity of the placental extracts is ACh.

The unidentified compound X in the term placenta was not hydrolyzed by alkali. It could not be separated from choline by ion-exchange chromatography using the ion exchange resin Amberlite CG-50. The source of this unidentified compound is not known, that is, whether it exists in placental extracts or whether it is formed by dealkylation from another compound.

There are unexplained significant differences in the concentrations of ACh from placenta to placenta. Some of these differences might be explained by the clinical histories of the mothers, the mode of delivery, the duration of labor, etc. It is quite possible that considerable amounts of ACh might have decomposed in the period between delivery of the fetus and of the placenta.

C. NATURE OF OCCURRENCE OF ACh IN HUMAN PLACENTA. Placental ACh is bound within membranes. When a human placenta was frozen for 48 hours and warmed to laboratory temperature, the entire amount of ACh decomposed. When the placenta was kept for 77 days at 4°C, about 30% of it remained in the tissues. The formation of ice crystals in the frozen tissue destroys the membrane structure and ACh is released. It is then hydrolyzed by maternal, fetal, and placental cholinesterase. Similar results with brain tissue have been reported in the literature (449). Freezing and thawing of brain tissue brings about the release of tissue-bound ACh, which is destroyed by cholinesterases. According to some estimations (59), a major portion of ACh-like activity (95%) in human placenta is in a bound form.

D. LOCALIZATION AND DISTRIBUTION OF ACh IN HUMAN TERM PLACENTA. ACh is localized in the villus tissue of the human placenta. There are 20 to 30 large villus trunks that correspond to the cotyledons (lobes) arranged in a circle around the umbilical cord (449). The concentric segments next to the umbilical cord and the peripheral segment have lower concentrations of ACh than the central concentric segments. Therefore, high ACh concentrations are found at positions where villi are localized. The ACh concentrations in the various sections of the placenta can be arranged in the following order: villus tissue > basal plate which contains parts of anchoring villi > chorionic plate. The surface areas of the trophoblast in the above sections can be arranged in the same order. The concentrations of ACh in floating villi and the basal plate are about 322% and 210%, respectively, of that of the chorionic plate.

The syncytiotrophoblast layer is fully developed by about 4 months and the cytotrophoblast disappears (461). At this time, high concentrations of ACh are found in the placenta. The fully developed villus contains three layers: syncytiotrophoblast,

connective tissue, and vascular fetal endothelium (461). In histological sections, certain granules can be localized in the syncytiotrophoblast by a special fixative (1% ammonium reineckate in 10% formaldehyde or 80% alcohol) that precipitates ACh and many other amines (479). These granules are localized along the free border of the syncytiotrophoblast. In electronmicrographs there are vesicles at the base of the microvilli of the syncytiotrophoblast (461). The surface areas of the syncytiotrophoblast have the following order: villus tissue > basal plate which contains anchoring villi > chorionic plate (461). The ACh concentrations in these tissues have the same order (396). Therefore, ACh is possibly localized in the syncytiotrophoblast. However, it has yet to be demonstrated that ACh is contained in vesicles.

E. SUBCELLULAR DISTRIBUTION OF ACh IN HUMAN PLACENTA. The subcellular distribution of acetylcholine (ACh) in villi dissected from human term placenta was studied using homogenization and differential centrifugation techniques by Olubadewo and Sastry (323) and Kau *et al.* (241). ACh in nuclear (P1), mitochondrial (P2), microsomal (P3), and high speed supernatant fractions were analyzed by pyrolysis gas chromatography. The percentage of recovery of ACh was about 83%. Although the absolute values of ACh in each placenta varied widely from one placenta to another, the subcellular fraction with the highest ACh content in all of them was the high-speed supernatant, S3. This fraction accounted for an average of 79% of the total ACh in the whole homogenate. Small amounts of ACh were found in fractions P1 and P2, which contained approximately equal amounts of ACh. The microsomal fraction, P3, contained only about half of the ACh content of either P1 or P2. The subcellular distribution of choline acetyltransferase was similar to that of ACh in the placenta. This pattern of subcellular distribution of placental ACh differs significantly from that commonly reported for the mammalian central nervous system (*e.g.*, adult rat brain). The subcellular distribution of ACh in the fetal rat brain is similar to that in the placenta (323). These studies (241,323) indicated that ACh storage granules in human placenta, if present, are immature and withstand the homogenization procedure. Alternatively, they suggested that ACh-storage granules comparable to the synaptic vesicles in the neuronal terminals of nervous tissues do not exist in the term human placental villus.

F. VARIATION OF THE CONCENTRATION OF HUMAN PLACENTAL ACh AS A FUNCTION OF THE GESTATION PERIOD. There is variation of ACh content with gestational age (396), and the highest concentration was found at about 22 weeks (wk) of gestation (nmol/g: at 9–12 wk, 129; 13–16 wk, 342 ± 31; 17–20 wk, 317 ± 32; 21–24 wk, 723 ± 63; 25–28 wk, 231; 29–32 wk, 249; 33–36 wk, 153 ± 15; 37–40 wk, 105 ± 7; 41–44 wk, 88 ± 5). The variation in choline acetyltransferase activity showed a pattern similar to that seen for ACh and remained the same whether choline acetyltransferase activity was expressed as per unit weight of tissue or of protein (42, 396). There was peak choline acetyltransferase activity at about 16 to 20 weeks of gestation and a 4-fold decrease in activity of parturition as well as at 9 to 13 weeks of development (174, 396). The development of the placental cholinergic system, as indicated by ACh and choline acetyltransferase, follows the development of the syncytiotrophoblast (461) during the first 6 months of pregnancy. The reasons for the decrease in choline acetyltransferase and ACh concentrations in the term placenta are not known. To obtain reliable values, it is necessary to collect the term placenta under careful conditions, and to follow the levels of ACh in placenta during the period between the delivery of the fetus and the delivery of the placenta.

G. VARIATION OF ACh CONTENT OF HUMAN PLACENTA UNDER VARIOUS CLINICAL CONDITIONS. Only limited numbers of studies (174, 382, 489) are available on ACh and choline acetyltransferase in placenta. In severe toxemias, ACh-like activity of placenta

has been reported to be increased (489). However, the exact gestation periods of these placentae are not known. Very low levels of ACh-like activity have been found in mild cases of toxemia. The levels of choline acetyltransferase in placentae of preeclamptic women are about one-third those of normal term placentae (174). Very low levels or total lack of ACh may have the same physiological effect as very high levels of free ACh, because high levels of ACh exhibit an antagonist effect, and low levels may not exert a significant effect at cholinergic sites.

As has already been discussed, the immature human placenta of the first trimester contains low levels of ACh and choline acetyltransferase (41, 174, 396, 472), and there is a relationship between the development of the human placenta cholinergic system and maturation of placental villi during the first 6 months of pregnancy. If ACh plays a significant role in maturation of placental villi, a deficiency of ACh should result in disturbance of maturation. The most impressive example of severe disturbance in maturation of placental villi is the hydatidiform mole. According to available information, hydatidiform mole contains only traces of choline acetyltransferase and only 6 to 7% of the concentration of ACh normally found in mature placental villus (382). Therefore, it seems possible that ACh may regulate the maturation processes or other maturation factors in placenta.

4. Release of ACh from Placenta and Factors Related to This Release. According to Chang and his coworkers (58, 61), ACh-like substances are released into the maternal blood side during the first 2 hours of perfusion of placenta with oxygenated saline. These studies have been confirmed by Raghavan and Sastry (355, 356). More detailed studies on the release of ACh from placenta were carried out by Sastry and his collaborators (324–326, 394) using isolated placental villus.

Isolated villus from human term placenta contained about 167 nmol of ACh/g when it was incubated in a muscle bath containing Krebs-Ringer bicarbonate buffer (pH 7.2–7.4) at 37°C, and ACh was released into the medium. This spontaneous release of ACh into the medium was linear with time and was about 35 pmol/g/min. ACh was not released when Ca^{++} was absent from the medium. Raising the Ca^{++} concentration in the Krebs-Ringer bicarbonate buffer from 2.34 to 4.64 mM, or adding L-nicotine (58μm) to the bath, increased the rate of release of ACh to 53, and 47 pmol/g/min, respectively. Nicotine did not exhibit any effect on ACh release in the absence of Ca^{++} in the medium. Both the rate of the spontaneous release of ACh and the nicotine-induced increase in the release of ACh were decreased by atropine (152 μM) and were not influenced by *d*-tubocurarine (30 μM). Depolarizing concentrations of potassium (16–63 mM) in the medium increased the rate of release of ACh. Cocaine, a known Ca-antagonist, decreased the rate of spontaneous release of ACh as well as the nicotine-induced release of ACh (326, 394). These observations indicate that: 1) Ca^{++} ions in the external medium are required for release of ACh; 2) Ca^{++} ions act as a link between the stimulation of ACh release and the final release of ACh; and 3) the effect of nicotine on placental release of ACh may be classified as the muscarinic type. It is not known if vesicular (231), contractile (345), or microtubular models of ACh release are applicable to placenta.

Enkephalins and endorphins are modulators of neurotransmitter release in nervous tissues (144, 265, 439). Recent investigations have indicated that human placental villus contains enkephalin- and endorphin-like activity (67 μg/g of leucine enkephalin equivalents) when analyzed by bioassay (397a). Enkephalin-like activity seems to have the same distribution as that of ACh in human placenta. Therefore, enkephalin-like peptides may regulate placental ACh release, or the release of enkephalin-like peptides from placenta into maternal circulation may regulate sensory transmission (or pain impulses) to the central nervous system from the uterus and vaginal

tract during child birth. Definite investigations are needed in this area.

5. Uptake of ACh by Human Placental Fragments. The fate of ACh released from human placenta is not well understood. Recent evidence has indicated that the parenchyma of the human placenta contains very little or no acetylcholinesterase (see section VIII A2). These findings raise questions as to the disposition of ACh which is possibly located in the syncytiotrophoblast cells of the placental villi from where it is released to exert its postulated physiological effects. One possibility is that the released ACh is hydrolyzed by the maternal plasma and erythrocyte cholinesterases, because placental villus is constantly bathed in the maternal blood. A second possibility is the reuptake of released ACh by the placental villus. An ACh uptake system, the physiological significance of which is not understood, has been well characterized in brain tissue (184, 280, 346, 411). An accumulation of ACh against a concentration gradient has recently been described in human term placenta (473, 474). This uptake system has a K_m of about 15 mM. The uptake of ACh by placental villus was competitively inhibited by choline (5×10^{-4} M), hemicholinium-3 (2×10^{-4} M), and morphine (10^{-3} M) (474).

6. Precursors for the Synthesis of ACh in Human Placenta. Acetylcholine is synthesized from choline and acetylcoenzyme A in the presence of choline acetyltransferase.

A. CHOLINE UPTAKE SYSTEM IN HUMAN PLACENTA. The importance of placental transfer of choline, a prominent constituent of lipid-soluble and several water-soluble metabolites including ACh, to meet placental and fetal needs is now well established. Choline-containing phospholipids cannot cross the placenta readily (30), and the ability of the fetal liver to synthesize the amine appears to be limited. Therefore, a source for this important lipogenic base may be the placenta itself. Or, choline may be produced in the maternal liver, the organ with the highest synthetic capability in the body (36), and the placenta may be involved in the transport of this choline. Free choline was rapidly taken up against a concentration gradient from extracellular water into the intracellular water compartment when human term placenta fragments were incubated *in vitro* (475). This choline is rapidly incorporated into several esterified products among which ACh is the most prominent.

According to the studies of Welsch (475), the human placenta does not have the capacity for the synthesis of choline *de novo*. In this apparent inability to synthesize choline, the cholinergic system of placenta is quite similar to brain tissue. With regard to the latter, the general consensus is that the brain is dependent on the supply of choline from a source that is probably phospholipid bound when it passes through the blood-brain barrier (8). Only one report has recently appeared claiming that brain tissue can perform methylation reactions leading to the synthesis of choline (244). As fas as the placenta is concerned, the lack of evidence for choline synthesis supports the view that the active choline uptake system may be the supplier of free choline for placental and fetal needs. An alternative route, which requires further experimental evaluation, would be the uptake of choline in a lipid-bound form or, less likely, in a water-soluble esterified form. If choline reaches the placenta in a phospholipid-bound form, then the base would have to be freed *in situ*. This process has been found to be very rapid and significant in rat brain (104). It has been observed that the free choline content of human placenta increases insignificantly upon storage at 4°C.

B. SOURCES FOR ACETYLCOENZYME A IN HUMAN PLACENTA. ATP citrate lyase is a cytoplasmic enzyme that forms acetylcoenzyme A in the presence of citrate, CoA, ATP, and Mg^{++}. Acetylcoenzyme A is one of the substrates for the formation of ACh by choline acetyltransferase. In order to evaluate whether citrate is a source of the acetyl group for the formation of ACh in placenta, Chaturvedi and Sastry (63) ana-

lyzed placental homogenates for ATP citrate lyase. The ATP citrate lyase activity of human placenta is 701 nmol of acetylcoenzyme formed per mg of protein per hr. It is not known whether there are other sources for the formation of acetylcoenzyme A in the placenta, *e.g.*, cytoplasmic acetate thiokinase.

7. Acetylcholine Receptor in Placenta. Several approaches have been used to delineate the cholinergic receptors in human placenta. The available evidence indicates that there are muscarinic receptors in human placenta. It is not known whether there are significant differences between muscarinic receptors in placenta and smooth muscle.

A. EFFECTS OF CHOLINERGIC AGONISTS AND ANTAGONISTS ON PLACENTAL ACh RELEASE. Discussed in section VIII A4.

B. EFFECTS OF CHOLINERGIC AGONISTS AND ANTAGONISTS ON AMINO ACID TRANSPORT. Discussed in section VIII A8D.

C. ACh-BINDING PROTEINS. Several studies have indicated that there are cholinergic receptors in human placenta. Attempts have been made to characterize the receptor on the syncytiotrophoblast. Although ^{125}I-α-bungarotoxin binds to the syncytiotrophoblast, this binding is nonspecific (241). Recent reports aimed at discovering the muscarinic receptor in placental villi *via* ^{3}H-quinuclidinylbenzilate (QNB) binding have yielded conflicting results (117, 480). However, some studies have indicated that a muscarinic-type cholinergic receptor is present in the human placenta. The muscarinic receptor of the placenta may not be of a classic type. It is suggested that the muscarinic receptors of placenta (M_2 type) may be slightly different from the classic muscarinic receptors (M_1 type). If this is true, more specific M_2-receptor antagonists have to be discovered before ACh-binding proteins from placenta can be isolated.

D. PLASMA MEMBRANE VESICLES FROM HUMAN PLACENTA. Although details have not been published, there is one preliminary report on the preparation of plasma membrane vesicles from the syncytiotrophoblast (118). These vesicles (0.1–0.2 μm in diameter) were separated by ultracentrifugation and identified by staining with iron hydroxide and 19- to 21-fold enrichment of 5′-nucleotidase activity in these membranes.

It was reported that these vesicles concentrated amino acids just like intact placenta (118). In view of the fact that intact plasma membrane is necessary for amino acid transport in placenta (372), these vesicles are possibly derived from intact pieces of plasma membrane.

The above vesicles had concentrated acetylcholinesterase activity. They bound QNB, a potent muscarinic agonist with an apparent K_d of 2×10^{-8} M. Nicotine-induced release of ACh from placental villus is blocked by atropine (326, 394). Therefore, it is tempting to conclude that QNB-binding sites represent muscarinic receptors. The microvilli on the syncytiotrophoblast have a tendency to retain erythrocyte ghosts adhered to its surface. One has to eliminate the possibility that QNB-binding to vesicles does not represent binding to erythrocyte ghosts. Although many problems have yet to be solved, a viable plasma membrane vesicle preparation would be a welcome advance in the area.

8. Effects of ACh on Various Parameters in Human Placenta. The major pharmacological effects of ACh in placenta include its influence on (a) placental blood vessels, (b) release of placental hormones, (3) phospholipid metabolism, and (4) amino acid transport.

A. EFFECTS OF ACh ON PLACENTAL BLOOD VESSELS. Acetylcholine (5–100 mg) usually has no effect or only a slight effect on the perfusion pressure of human placenta (78, 110, 115). In an occasional placenta, it causes weak dilation or a pronounced constriction enhanced by physostigmine and abolished by atropine. Norepinephrine causes constriction of placental blood vessels and raises perfusion pressure. This effect is sometimes enhanced by cocaine and always abolished by ergotoxine (115). It is not known whether or not ACh

releases placental catecholamines, which increase perfusion pressure, in an occasional placenta. This becomes more important in view of the fact that several endogenous substances cause an increase in perfusion pressure in isolated placentae (Table 5). For example, histamine and serotonin are potent vasoconstrictors of placental vessels (78, 110, 115, 133, 134).

Both known transmitters of nervous excitation, norepinephrine and ACh, have effects on the tone of the placental vessels, those of norepinephrine being the more regular. The effects show that these transmitters are not without action on plain muscle cells that have never been innervated. They do not, on the other hand, exclude the possibility that connection with nerve endings may confer on muscle cells that acquire it, a sensitivity of higher degree and greater specificity for one or the other of these substances. Indeed, the relatively weak and somewhat variable reaction exhibited by the placental vessels might well be regarded as representing a primitive ability to respond to these transmitters. In somatic arteries that acquire a nerve supply, this ability to respond to norepinephrine would accordingly not be evoked as an entirely new property by union with nerve fibers of the sympathetic system, but would, nevertheless, be greatly enhanced thereby and given a definite direction. Alternatively, these transmitters in the noninnervated cells may serve some first messenger function, other than transmitter excitation, which is not known.

In some experiments, ACh showed a definite constrictor action on the placental vessels—an action that, like its other peripheral effects, was readily abolished by atropine. It is worthy of note that such a vasoconstrictor action of ACh in small doses has elsewhere been regularly observed on the pulmonary vessels of the rabbit (115); and it is tempting to correlate this similarity of response with the fact that the arteries of both the placenta and the lungs are conveying venous blood, and are structurally adapted to withstand only moderate pressures. There is a similar resemblance between the response of the placental and the pulmonary vessels to adenosine, which causes constriction of both, in contrast to its general vasodilator action. These vasoconstrictor effects of ACh and adenosine on

TABLE 5

Effects of biogenic amines and other endogenous substances on perfusion pressure in isolated human placenta

Substance	Dose[a]	Effect (frequency of occurrence, %[b])			Intensity of Major Effect
		Constriction[c]	Dilation[c]	Weak or Absent	
	μg				
1. Acetylcholine	2–500	11	27	56	±[d]
	100	80		20	1–5[e]
2. Epinephrine	2–500	73		27	+++[d]
	10–500	69	18	13	3–12[e]
3. Norepinephrine	10–500	90		3	3–5[e]
4. Histamine	5–20	50		50	+++[d]
	10–100	85	11	4	14–65[e]
5. Serotonin	2–5	97		3	21–77[e]
6. Adenosine	100–600	67	33		++[d]
7. Vasopressin	2–10 I.U.	43		57	+[d]
8. Prostaglandin	0.2–2 units	100			+++[d]

[a] Unless otherwise stated.

[b] Frequency of occurrence indicates that the number of placenta in which the effect will be observed in a total of 100 full term placenta.

[c] Constriction and dilation are judged from the increase and decrease in perfusion pressures.

[d] Data summarized from von Euler (115).

[e] Data summarized from Ciuchta and Gautieri (78). Figures indicate mm Hg.

the placental vessels are not seen in all experiments. Both, on occasion, have caused vasodilation, and the question arises whether their actions, in causing an increase of the resistance to perfusion, are necessarily, or entirely, upon the blood vessels. If the placenta contained extravascular contractile tissue, responding to these substances by contraction, its reaction might cause an obstruction to the flow simulating vasoconstriction, and even, perhaps, masking a concomitant relaxation of the vascular walls themselves. The contractile properties of the trophoblast cells and the nature of contractile proteins in the trophoblast, and the nature of receptors on the trophoblast membrane should be better understood before definite statements about the effects of ACh and norepinephrine on placental vessels are made.

B. RELEASE OF PLACENTAL HORMONES BY ACH. Human placenta is a source of chorionic gonadotrophins and steroid hormones. Since the development of the placental cholinergic system follows the development of the syncytiotrophoblast, it would be interesting to determine the release of steroid hormones by ACh, not yet investigated.

Although the cytotrophoblast, the source of gonadotrophin in the placenta, is fully developed in the first 3 months of gestation, some cytotrophoblastic cells are left in full term human placenta. It has been reported that ACh stimulates placental release of chorionic somatomammotrophin *in vitro* (176).

C. EFFECTS OF ACH ON PHOSPHOLIPID METABOLISM IN HUMAN TERM PLACENTA. The modulating effect of ACh on phospholipid metabolism has been demonstrated in nervous tissue (brain, ganglia) and glandular tissues (*e.g.*, avian salt gland, parotid gland, pancreas) (106, 207, 278, 363, 401). In general, the addition of cholinomimetic drugs results in an increase in labeling with radioactive inorganic phosphorus of phosphatidic acid (PA) and its derivative, phosphatidyl inositol (PI). The functional significance of this phenomenon is not understood, but the ACh-stimulated turnover of PA and PI ("the phospholipid effect") has been implicated in the control of permeability in excitable membranes (106).

The human placenta has prominent functions as an endocrine gland. This tissue is not innervated (130), yet it contains high concentrations of ACh and choline acetyltransferase, which synthesizes ACh. The physiological function of placental ACh is still obscure but on the basis of clinical observations and the effects of certain pharmacological agents on fetal growth, it has been speculated that its presence may be related to permeability and transport processes across the placental barrier (15, 256). The formation of a phospholipid-amino acid complex has been reported to provide a means of transfer of amino acids from mother to fetus (15); the phase of rapid growth during human fetal development coincides with the highest activity of choline acetyltransferase and the highest ACh concentrations. In view of these observations, the effects of ACh on phospholipid metabolism in human placenta are of interest. The incorporation of $^{32}P_i$ into term placental polar phospholipids has been measured in the presence or absence of 1×10^{-4} M exogenous ACh with particular attention to phosphatidic acid, phosphatidyl inositol, and phosphatidyl choline. There are no changes in the intensity of labeling of any of the phospholipids because of the presence of ACh. No studies are available on the placenta of gestation period 3 to 6 months, the period of the development of the syncytiotrophoblast. Further, these studies may not provide a correct picture of the effects of exogenous ACh. If ACh has any effects on phospholipid synthesis, these effects are already maximally stimulated by endogenous ACh. Atropine concentrations (10^{-5} M) used in this study were not adequate to block completely the large concentrations of endogenous ACh present in the placenta and the ACh that is continuously synthesized in the placenta. In the well known *in vitro* systems, about 15 molecules of atropine are

required to block 1 molecule of ACh, because of its large volume of distribution and nonspecific binding (373). It would be interesting to find out the effects of exogenous ACh on phospholipid synthesis when the synthesis of endogenous ACh is blocked. Further, the inability of exogenous ACh to alter phospholipid metabolism does not rule out the possibility that ACh has a function in the control of transport processes and membrane permeability of the placental membranes by a mechanism unrelated to their phospholipid turnover.

D. ANTAGONISTS OF ACh AND AMINO ACID UPTAKE BY ISOLATED PLACENTAL VILLUS. Isolated villus from human term placenta contains about 167 nmol of ACh/g of wet tissue and releases about 35 pmol/g/min of ACh when it is suspended in Krebs-Ringer bicarbonate buffer at 37°C (326, 394). This tissue has conveniently been used to measure the uptake of amino acids by placenta. α-Aminoisobutyric acid is a nonmetabolizable amino acid and can be used as an indicator of active amino acid transport (174, 372, 373, 437). Chronic doses of nicotine or smoking that modify ACh output from human placental villus are known to retard fetal intrauterine growth (450, 462). Since one of the functions of placental villi is nutrient transport, and since it has not been possible to obtain ACh-free villi, the effects of cholinergic blockade using high concentrations of ACh (2×10^{-3} M), phospholine (5×10^{-4} M), nicotine (2.5×10^{-3} M), and atropine (10^{-4} - 5×10^{-4} M) on active uptake of a α-aminoisobutyric acid have been studied by Rowell and Sastry (372, 373) to explain their antigrowth effects. High concentrations of ACh and nicotine decreased the rate of uptake of α-aminoisobutyric acid by 34 and 41%, respectively. Atropine inhibited the uptake of α-aminoisobutyric acid by 29 and 61% at concentrations of 10^{-4} and 5×10^{-4} M, respectively. If all of the ACh were released from the syncytiotrophoblast, the concentration of ACh in the medium would be about 1.67×10^{-5} M. At the highest concentration of atropine used, the concentration of the active antagonist, D(−)S-hyoscyamine, in the medium was 2.5×10^{-4} M which depressed the uptake of α-aminoisobutyric acid by about 61%. Possibly, these high concentrations of D(−)S-hyoscyamine were required for blocking heroic concentrations of endogenous ACh.

Phospholine (an irreversible cholinesterase inhibitor) at 7×10^{-6} M increased the uptake of α-aminoisobutyric acid by 20%, but it decreased the uptake of aminoisobutyric acid at higher concentrations with or without exogenous ACh. Mecamylamine (10^{-4} M) and *d*-tubocurarine (10^{-4} M) did not influence the uptake of α-aminoisobutyric acid. Nicotine increased the release of endogenous placental ACh, while atropine decreased ACh release (326, 394). These observations indicate that endogenously released ACh exhibits a muscarinic effect on the placental villus and facilitates the uptake of amino acids. Blockade of the facilitating effects of ACh on amino acid uptake by placenta for long periods during pregnancy may result in a retardation of fetal growth.

E. INHIBITORS OF CHOLINE ACETYLTRANSFERASE AND AMINO ACID UPTAKE BY ISOLATED PLACENTAL VILLUS. There were no suitable selective inhibitors of ACh until the discovery of 2-benzoylethyltrimethylammonium and related compounds (64, 368). In only one type of tissue, human trophoblast, has the influence of choline acetyltransferase inhibitors on amino acid (α-aminoisobutyric acid) uptake been studied.

Potent choline acetyltransferase inhibitors depressed amino acid uptake by placental villus. There was a direct relationship between the degree of inhibition of choline acetyltransferase and the degree of inhibition of α-aminoisobutyric acid uptake by choline acetyltransferase inhibitors (371). These observations indicate that the placental ACh system is linked to the placental amino acid uptake by unknown steps.

9. Placental ACh and Transport of Chemicals Across Placenta as a Function

of Gestation Period. There is a variation in the ACh concentration with gestational age of the placenta. The highest values for ACh concentration were found during 21 to 24 weeks of pregnancy. The variation in choline acetyltransferase activity showed a pattern similar to that seen for ACh (see section VIII A). There seems to be an inverse relationship between the variation in the levels of placental acetylcholine and choline acetyltransferase and variation in the capacity of placenta to transport substances as a function of gestation period (174).

There are two principal stages in prenatal development, an embryonic period (1–2 months) and a fetal period (2–9 months) (461). Neither the syncytiotrophoblast nor the placental cholinergic system is fully developed during the embryonic period. Many substances (*e.g.,* α-aminoisobutyric acid, 170, 174; diphenylhydantoin, 327) cross the placental barrier with relative ease (92, 145). The degree of the teratogenic sensitivity of the human fetus to chemicals reaches the highest levels during this period (145). During the first part of the fetal period (2–6 months), the organs undergo little more than maturation (histogenesis) at the histological level. The size of the human fetus increases by about 250 times during this period. Chemicals do not cross the placental barrier as easily as they did during the embryonic period, and their transport seems to be regulated. The placental cholinergic system is fully developed during this period. During the latter part of the fetal period, functional maturation of the fetus is achieved and the placental levels of ACh and choline acetyltransferase have decreased. Chemicals do seem to cross the placental barrier more easily than they did during the midgestation period (282, 436). These observations indicate that the placental cholinergic system may play a significant role in the regulation of the transport of nutrients and chemicals across the syncytiotrophoblast and thereby regulate fetal growth. Studies on the interrelationships between placental transport and fetal growth during the manipulation of the placental cholinergic system using pharmacological agents may provide some answers for the role of ACh in the placenta.

10. Placental ACh, Drugs of Abuse, and Intrauterine Fetal Growth Retardation. There are several drugs of abuse that interfere with placental ACh and are known to cause intrauterine growth retardation in experimental animals and humans (462). Morphine is known to decrease ACh output in the peripheral nervous system (265). Although its actions on human placental ACh are not known, morphine is known to induce fetal growth retardation in humans (450). Nicotine in small doses increases the release of human placental ACh, and, in high doses, it decreases ACh release (324–326). Nicotine and smoking are implicated in intrauterine fetal growth retardation in man (268, 462). Intrauterine fetal growth retardation among cocaine addicts is not well documented. However, it is known that cocaine interferes with Ca^{++} movements and depresses ACh release in peripheral tissues. All available evidence indicates that protein calorie malnutrition is one of the primary insults leading to intrauterine fetal growth retardation (34). Regulation of the amino acid environment in the mother or in the fetus is not significantly affected by smoking (491). However, amino acids for fetal protein synthesis have to be transported from maternal blood to fetal circulation through the placenta, and nicotine decreases the uptake of amino acids by human placental villus. There is no information on the effects of morphine and cocaine on amino acid transport across human placenta. However, in view of their effects on ACh release, they may depress amino acid transport across placenta.

B. Monkey Placenta

Term placentae of several species have been investigated by Hebb and Ratkovic (183) for choline acetyltransferase activity. These include monkey (*Macacus rhesus*), mongoose, lemur, horse, cow, sheep, goat, pig, hamster, cat, rabbit, guinea pig, and

rat. None of these placentae contain choline acetyltransferase except that of monkey.

Term placentae from rhesus monkeys (*Macaca mulatta*) have been investigated in detail by Welsch (476) for their contents of ACh and choline. In this study, choline acetyltransferase was measured by a radiometric assay in the presence of 1-^{14}C-acetylcoenzyme A and choline and distinguished from other acetyltransferases by a differential assay involving acetylcholinesterase or selective ion pair extraction of 1-^{14}C-ACh with tetraphenylboron. At 150 days of gestation, the rhesus monkey placenta synthesized about 4 μmol of ACh/g of wet tissue/hour. Choline and ACh were determined with a radiochemical method based on the phosphorylation of free choline by choline kinase in the presence of ^{32}P-ATP. Tissue levels of choline ranged from 737 to 3892 and ACh from 8.8 to 29 nmol/g of wet tissue.

C. Cow Placenta

Cow placenta contains large quantities of ACh-like activity (185). Its content is highest in membranes, medium in cotyledons, and minimal in the cord. The amniotic fluid of the cow contains ACh-like activity, as does the uterine muscle, during the whole gestation period. Just as in the human placenta, this activity in different parts of cow placenta rises during the progress of pregnancy. However, according to Hebb and Ratkovic (183), there are no measurable choline acetyltransferase activities in cow placenta. Therefore, questions arise about (a) the source of ACh-like activity in cow placenta, and (b) the stability of cow placental choline acetyltransferase, if any. In view of these controversial findings, reinvestigation of cow placenta is necessary.

D. Rabbit Placenta

Only negligible amounts (< 0.1 μg/g) of ACh-like substance were found in the placenta of rabbits by Chang and Gaddum (60). Similarly, only negligible amounts of choline acetyltransferase were found in rabbit placenta by Hebb and Ratkovic (183). The conclusions reached by these authors, which took into consideration the sensitivity of their methods, did not exclude the possibility of the occurrence of ACh-like substances other than ACh, and choline acetyltransferase-like enzymes with properties different from those of human placental choline acetyltransferase. The content of ACh-like substances has also been investigated by Reynolds and Foster (358) in the placenta and uterus of the gravid and pseudopregnant rabbit. Pregnancy comes to an end in the rabbit on the 32nd day and the placenta can be collected easily at different gestation periods. According to Reynolds and Foster (358), an ACh-like substance (0.44 μg/g) is found in the fetal placenta of the rabbit on the 16th, the 22nd, the 28th, and the 31st days of pregnancy. However, their data were too few and too scattered to assess variation of ACh-like substance with gestation period. The action of this ACh-like substance was potentiated by physostigmine on the frog rectus abdominis muscle.

An ACh-like substance has been found in the rabbit uterus throughout the course of pregnancy (358), and it increases in the presence of estrogens (359). It is not known whether estrogens increase actual ACh content or potentiate the effect of ACh in the bioassay preparation used for the estimation of ACh. This ACh-like substance was found on the average in lowest concentration in midpregnancy, intermediate in concentration on the 22nd and 28th days of gestation and during estrus, while on the 31st day it was present in more than twice the concentration (1.36 μg/g of uterus) found on any preceding day of pregnancy. On the 6th and 16th days of pseudopregnancy, the concentration of the ACh-like substance in the uterus was about equal to that found in the uterus during estrus and most of pregnancy. These investigations have not been repeated by others.

E. Rat and Mouse Placentae

Rat and mouse placentae were analyzed by Stevens *et al.*(446) for choline acetyl-

transferase and acetylcholinesterase activities at various periods of gestation. Choline acetyltransferase activity was present at all gestation periods studied with peak activity of 71 nmol of ACh synthesized/g of tissue/hour on day 14 in the mouse and 50 nmol of ACh synthesized/g of tissue/hour on day 15 in the rat. Acetylcholinesterase activity was present throughout gestation, peaking at 20 μmol of ACh hydrolyzed/g of tissue/hour on days 18 to 19 in the rat and 39 μmol of ACh hydrolyzed/g of tissue/hour on day 17 in the mouse. As determined by gas chromatographic methods ACh was present at all periods studied. Development of the enzyme systems in rodent placentae corresponds with that reported for the human placenta at similar developmental stages. The development and gestational dependency of this cholinergic system supports the concept of a cholinergic regulation of fetal growth and placental transport. Inhibition of placental acetylcholinesterase by paraoxon significantly depressed placental uptake and transport of the model substrate amino acid, α-aminoisobutyric acid.

The existence of choline acetyltransferase in mouse placenta has been questioned (477) in view of the high concentrations of carnitine in these placentae. Carnitine may act as a weak substrate for choline acetyltransferase. The degree of contamination from erythrocyte acetylcholinesterase was not evaluated in these studies. For the validation of these studies, further information is necessary regarding: 1) the specificity of carnitine acetyltransferase that is present in these placenta; 2) analysis of placenta for choline acetyltransferase after the homogenates have been dialyzed; 3) stability and specificity of choline acetyltransferase activity in a dialyzed preparation; and 4) the degree of contamination of these placental acetylcholinesterases by erythrocyte acetylcholinesterase.

F. Species Variation in the Placental Cholinergic System

According to available information, the two species in which the placental cholinergic system has definitely been found are the two higher primates, man (396) and monkey (183, 476). The fact that no choline acetyltransferase is found in placentae of several other species might be due to species differences in the molecular forms and the stabilities of the enzyme, which vary from species to species (183, 286, 484). Similarly, absence of ACh cannot be regarded as a definite proof that the tissue cannot synthesize ACh. For example, human and bull spermatozoa have the capacity for the synthesis of ACh, but they do not store ACh (28). More sensitive methods and novel experimental techniques will be necessary to establish the absence of cholinergic system in placenta of species other than primates. In spite of these observations, human placentae and possibly monkey placentae are unique in that they have the capacity to store ACh. In primates, there is a selective transfer of antibodies across the placental barrier (13), whereas, in other species such transfer occurs by other routes (33). Further, the occurrence of fetal intrauterine growth retardation with teratological effects seems to be more prevalent in man than in lower species. Possibly, these distinctions provide clues to the function of ACh in higher primates. Further work is necessary on the placental cholinergic systems of various species before definite statements can be made about species variation.

IX. Cholinergic System in Plants

The primary focus of this review is on cholinergic systems of animal tissues. However, some data are available that indicate that ACh has functions in plants which are analogous to non-neuronal functions in animals.

The fluid from the hairs of the nettle plant, *Urtica urens*, contains ACh (111a). It is uniformly distributed in leaves, outer cortex of both stem and main root, and rootlets. An enzyme which has properties similar to those of ox brain choline acetyltransferase is present in leaf homogenates

of the nettle, *Urtica dioica L.* (15a). Young as well as old leaves synthesize ACh.

Acetylcholine and acetylcholinesterase have been found in the roots of the mung bean, *Phaseolus aureus* (227a). Acetylcholine is present in all organs of both light- and dark-grown seedlings. The highest concentrations were found in tissues containing active growing points—buds and secondary roots. Red light caused an increase in the efflux of ACh from secondary root tips and a significant increase in the endogenous ACh. Far red light reduced the latter to a level comparable to that in the dark. Acetylcholine, given in the dark, substituted for red light in reducing the formation of secondary roots, inducing H^+ efflux and causing the root tips to adhere to a negatively charged glass surface. Atropine inhibited the latter phenomenon.

The phytochrome-mediated response to red light in roots is rapid utilization of ATP pools; far red light inhibited this utilization (491a). Acetylcholine in the dark promoted utilization of ATP pools. According to Yunghans and Jaffe (491a), ACh facilitates ion transport at many target sights and modifies the mitochondrial membrane, which results in oxygen consumption and ATP utilization. The bond energy of ATP may then be utilized for active transport of monovalent cations. Far red light induces the destruction of ACh or prevents ACh from reaching the target sites, thus reversing the effects of red light on respiration. Plant growth retardants related to (+) limonene inhibit acetylcholinesterase and increase the levels of endogenous ACh (359a), which may be high enough to block the cholinergic receptors involved in the growth processes of secondary roots. More research is needed to clarify the relationships between ACh, ATP utilization, ion transport, and growth in plants.

X. Possible Roles of ACh in Non-nervous Tissues

In many organisms, hormones and neurotransmitters serve as first messengers to provide intercellular communication. While hormones provide communication over long distances, transmitters operate over very short distances. The transmitter function for ACh in nervous tissues is well established. The evolutionary appearance of ACh preceded that of the nervous system. Also, ACh occurs in several tissues, where it serves no apparent neural function. At least, in some tissues, the ACh system is fully developed during maturation, growth, and possibly during regeneration. ACh synthesis does seem to decrease during aging in some nervous and non-nervous tissues (395, 467). These observations should provide some clues for evaluating the role of ACh in non-neuronal functions. It may be more rewarding to consider the role of ACh in non-nervous tissues as similar to that of a messenger. Although different roles are listed below for ACh in nervous tissues, several of these may turn out to be linked to one another when more knowledge becomes available.

A. ACh as an Environmental Messenger

Recently, Tomkins (459) postulated "The Metabolic Code," a model for the evolution of biological regulation and the origin of hormone-mediated intercellular communication. According to this hypothesis, neurotransmitters are derived from amino acids and amino acid metabolites. Transmitters might have acted in primitive cells as intracellular symbols representing changes in amino acid concentration. Eventually, these symbols are possibly utilized in short-range intercellular roles by primordial nerve cells (291).

It is possible that non-nervous cells like bacterial cells that release ACh into the culture medium utilize ACh to send information regarding intracellular amino acid (or other nutrient) concentrations to the external side of the membrane. Then ACh may depolarize the membrane and activate the membrane-pores which expose the transport systems to their substrates. It is implicit that an environmental messenger acts on the membrane of the cell from which it is released. There is some evidence

that ACh may act as an environmental messenger in cultured fibroblasts (section V A) and human placental trophoblast (Section VIII).

B. ACh and Cell-Cell Communication

It is known that ACh is a chemical messenger (or transmitter) from one nerve cell to another nerve cell or to muscle cells or secretory cells of various types. In all of these cases, one of the cells is a nerve cell. The transmitter is stored in the synaptic vesicles in the nerve cell. There is no evidence to indicate that ACh may be involved in cell-cell communication when both cells are non-nervous in origin. In cultured fibroblasts (section V A), cell-cell communication seems to be noncholinergic.

C. ACh as a Local Hormone

A local hormone is produced precisely when and where it is needed, exerts its specific regulatory effect, and is rapidly destroyed. ACh satisfies the above requirements to be classified as a local hormone in several tissues. It appears both to be synthesized by and to act on cardiac and smooth muscles in the maintenance of excitability and rhythmicity. Production of hyperpolarization and depolarization are probably the primary actions on the membranes of the above two types of cells respectively. However, the sequence of events linking excitability and rhythmicity to hyperpolarization and depolarization are not known.

ACh may have a local hormonal role in several ciliated membranes. ACh can modify the movement of cells, and its associated enzymes are present in a variety of cells that respond to ACh. The generalization, although obviously at the same level as our knowledge concerning the function of ACh in the nervous and neuroeffector systems several decades back, is encouraging nevertheless. However, the functional roles of ACh in ciliary and flagellar motility as well as in other types of cellular movement are still not clear, and a generally agreeable theory of flagellar and other cellular motility phenomena is not yet forthcoming. It is necessary, not only to obtain more evidence to support the hypothesis that the ACh system has important functional roles in cellular movement, but also to know how the cholinergic agents modify the detailed patterns of the movement. The transport of particles on the surface of ciliated epithelium depends on the various complex waves of ciliary motion on the lining cells. The terminology and identification of these waves was reviewed by Knight-Jones (247). The initiation and coordination of these waves depend on the interaction of a host of complex factors that include mechanical interaction, intracellular and intercellular communication, as well as neurohumoral influences (246, 435). These, in turn, are dependent on the initiation, bending, propagation, etc. within the individual cilium or flagellum, which is also responsible for translational movement in unicellular systems like the spermatozoa, some protozoa, and bacteria (38, 154, 314, 332). The gross effect of ACh could be due to some specific actions on one or more of these parameters or on some other metabolic processes that are coupled to the motile system. Of particular importance are the converging views of experts, who have studied various aspects of cell motility, that the basic events underlying movement in the diversified biological systems are likely to be similar. There is general agreement that ciliary motion is regulated in one way or another by some humoral mechanism (246) and bioelectric and electrolyte transport phenomena are likely to be involved (107).

D. ACh and Permeability

Highly reasonable proposals have been put forward stating that ACh may control passive permeability or active transport in several tissues such as placenta (174) and erythrocytes (333). However, considerably more work has to be done before these proposals can be firmly established. Two especially provocative proposals, are: 1) ACh regulates active transport of sodium ions and positively charged organic mole-

cules by a phosphatidic acid system in several glandular and nervous tissues (207, 251); and 2) ACh regulates active transport of amino acids in placenta (174, 371–373). Now it is clearly established that there are definite transport systems for ions (434) and amino acids (174). In the former case, phosphatidic acid is considered as a carrier for the active transport of sodium across the membrane. In this latter case, ACh secretion-stimulation and transport coupling have been postulated. The link between ACh stimulation of the receptor on the membrane and the transport is considered to be the alterations produced in the membrane exposing the carriers to their substrates (326). Nothing is known about the molecular nature of membrane alterations. The most exciting development in this area is the inhibition of amino acid transport by specific choline acetyltransferase inhibitors (371). However, these investigations are still only in preliminary stages.

From an evolutionary point of view, it can be surmised that the earliest function of ACh in primitive organisms is probably the modification of the passage of various substances across cell membranes. ACh may also play a similar role in the developing tissues. With the development of structural complexity of cellular membranes in accordance with specialized functions in higher organisms or completely developed tissues, the early role of ACh may be retained with varying degrees of specificity in some tissues or be masked in other tissues. In highly developed specialized tissues such as nervous tissue, the early or primitive function is possibly masked by the specialized function, namely chemical transmission (see also section V B).

E. ACh and Trophic Factors

According to available evidence, ACh as well as unidentified "trophic factors" may be involved in limb regeneration (see section IV E). In the regenerating limb, higher ACh levels and lower cholinesterase levels than those in the normal limb were found (427). With the differentiation of the regenerate, the activity of cholinesterase increases and ACh decreases. This relation of high ACh content and low cholinesterase activity during regeneration suggests that ACh may be a neurotrophic agent. Unidentified "trophic factors" other than ACh are found in nervous tissue (429). While ACh seems to support regeneration, it does not substitute for "trophic factors." This indicates that ACh may be involved in the release and regulation of trophic factors during regeneration. Since regeneration occurs in aneurogenic limbs, it would be interesting to find out the variations in ACh and other components of the cholinergic system during regeneration in aneurogenic limbs in salamander and related species or the regeneration of mammalian liver, which contains only a few nerve fibers.

F. ACh, Maturation, and Development

There seems to be a role for ACh during maturation and development. The cholinergic system is fully developed in tissues that undergo complete maturation. These relationships in erythrocytes (section VI A), human trophoblast (section VIII), and spermatozoa (section VII) have already been discussed. However, it is not known whether ACh itself is the maturation factor or if ACh action is linked to the action of other unidentified maturation factors.

G. ACh and Growth

The concentration of ACh increases in several developing tissues and decreases in fully developed tissues. Two proposals have been made: 1) ACh regulates amino acid transport and other nutrients; and 2) ACh stimulates protein synthesis. Some aspects have already been discussed in sections IV D and VIII A8D. In several of the studies it was difficult to dissociate the effects of ACh on amino acid transport from direct effects on protein synthesis (80, 150–152, 154). A growth-hormone-like effect of ACh in reducing nitrogen excretion following glycine loading *via* the intraperitoneal route in unanesthetized rats (150), and an increase in the incorporation of labeled glycine into

isolated rat diaphragm similar to the effect of insulin (151) have also been demonstrated. Several studies have used organophosphorus compounds to measure ^{14}C-lysine incorporation into whole brain proteins. In these studies, increase in protein synthesis is considered to be compensatory to accommodate the replacement of irreversibly inhibited enzyme (cholinesterases) proteins (79).

There is only one convincing study in which it has been demonstrated that specific choline acetyltransferase inhibitors inhibit the uptake of a nonmetabolizable amino acid, α-aminoisobutyric acid, by isolated placental villus (371, 373). However, there is no information on the effects of choline acetyltransferase inhibitors on protein synthesis. Studies on the effects of choline acetyltransferase inhibitors on protein synthesis by tissues that are capable of synthesizing ACh, but do not store ACh, are promising and will be rewarding.

XI. Conclusions and Scope of Future Investigations

From the foregoing review it is evident that one or more components of the cholinergic system are present in a variety of cells. Several types of cells other than nerve cells have the capacity for synthesizing ACh. These cells can be divided into two types—those which have both synthetic and storage capacities (*e.g.*, nerve cells, human trophoblast), and those which have synthetic capacity only (*e.g.*, sperm cells, fibroblast, erythrocyte). Several types of cells release ACh into the physiological medium in which they are incubated. In several types of embryonic cells, one or more components of the cholinergic system have been detected before innervation. ACh may play a significant role, different from its well known function as a chemical transmitter, in the embryonic and developing cell. Several reasonable proposals have been forwarded regarding the function of ACh in non-nervous cells or non-neuronal functions of ACh (see section IX). In all of these proposals, it is implicit that ACh acts at receptors located on the plasma or intercellular membranes, and may serve as a messenger. However, little is known about the nature of this messenger function. Considerably more work is necessary before a unitary function for ACh in non-nervous tissue can be postulated. There are many promising areas worthy of experimental investigation.

Although the various components of the cholinergic nervous system have been characterized, the components of cholinergic systems in non-nervous and embryonic cell systems have not. It has often been assumed that these systems are similar in their chemical and functional characteristics, but this need not necessarily be true. For example, the choline acetyltransferases in embryonic heart, spermatozoa, and the nerve cell need not be similar. There may be different types of choline acetyltransferases whose substrates may vary, or there may be two types of choline acetyltransferases, specific and nonspecific with respect to either choline or acetylcoenzyme as a substrate. The stability of these enzymes from nervous and non-nervous sources may vary. For example, the choline acetyltransferases from embryonic tissues may not be stable during isolation procedures, or their behavior toward substrates, or inhibitors, or both may change during homogenization and separation procedures.

In the case of cells that can synthesize ACh but lack membrane stores, ACh synthesis, the stimulation of the receptor by ACh, and the hydrolysis of ACh by cholinesterases should be closely linked and may be localized within the same cell compartment. Such a control system may be delicate and its components may be destroyed during homogenization and isolation. However, such a system should be very sensitive to inhibitors of choline acetyltransferase, because there is no preformed ACh. However, no studies have been reported on the effects of choline acetyltransferase inhibitors on such cell systems and their functions except on human sperm motility (384; see also section VII F2). One of the problems

in this area may be the lack of suitable inhibitors of choline acetyltransferase. Several aspects of the role of ACh can be investigated using choline acetyltransferase inhibitors. These include the role of ACh in maturation, regeneration, differentiation, and development of various tissues, cell permeability, and mobility of cells.

Several aspects of the structure of cilia, flagella, and other dynamic components of cells are known (214, 440). The occurrence of microfilaments and microtubules in several cells capable of movement suggest that surface receptors, microfilaments, and microtubules may be working as a unique set of interacting structures mediating cell movement and biological response of the cell to external ligands including other cells. The question should be asked whether ACh is the natural ligand and whether cholinergic receptor-ACh interaction initiates different levels of interaction: receptor-microfilament, microfilament-microtubule, microfilament-enzyme, or cofactor interaction. Investigations on these aspects may provide information on cell mobility, automaticity, and rhythmicity.

There is considerable evidence to indicate that ACh may play a significant role in the regulation of trophic factors involved in the regeneration of tissues in salamanders and related species (section IV E). Regeneration occurs also in mammalian skin, skeletal muscle, and liver. However, no information is available on the role of ACh in the regeneration of mammalian tissues.

Progress in this area may depend on the study of the right systems and use of the right techniques. During the past decade, biochemical techniques have become available for studying cholinergic mechanisms. The next 10 to 20 years should be exciting since several scientists are beginning studies on interesting biological roles of ACh in embryonic and non-nervous tissues.

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