ANTICHOLINESTERASE DRUGS

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INTRODUCTION. The pharmacological actions of "anti-cholinesterase drugs" can be considered only after certain limitations have been placed on the definition of the compounds in this category. The first limitation is concerned with the nature of the enzyme inhibited. The term "cholinesterase" (ChE), originally applied to any acetylcholine (ACh)-splitting enzyme of the blood or tissues, has been shown to be too inclusive. There are several enzymes which hydrolyze ACh, and their substrate specificities and susceptibilities to inhibition by different agents vary greatly. Moreover, the physiological significance of many of them in respect to the hydrolysis of ACh is highly questionable. Unfortunately, tests of the anti-ChE activity of drugs have been conducted largely with the ACh-splitting enzyme of mammalian plasma rather than with tissue enzymes. Secondly, literally hundreds of compounds have been reported as more or less potent inhibitors of one or another type of ChE, and many of their pharmacological actions have been attributed to this property. It is generally a wise procedure to adhere to the old pharmacological dictum that no drug has a single action. Undoubtedly, the property of many of the drugs mentioned below to inhibit ChE constitutes a relatively unimportant side-action. On the other hand, potent and specific inhibitors of ChE may exert direct actions on effector cells. An inhibitor combines with an enzyme by virtue of an affinity between certain active groups of the enzyme and inhibitor molecules. Inasmuch as ACh is able to combine with either the enzyme molecule or some "receptor group" of the effector cells, it is likely that enzyme and cell receptor have certain chemical or physical properties in common. It is therefore reasonable to assume that an enzyme inhibitor might also combine with the receptor groups of the effector cells, leading either to the initiation of a response similar to that evoked by ACh or to cholinergic blockade. Specific examples of drugs which appear to act by these mechanisms are cited below.

The present review will be largely confined to a discussion of the actions *in vitro* and *in vivo* of a limited group of compounds which are potent inhibitors of specific ChE, namely, the alkaloids physostigmine, neostigmine and their congeners, and an expanding group of alkyl phosphates. For orientation and background the types of ChEs and their distribution will first be considered.

Any discussion of anti-ChE drugs is bound to touch upon the controversial issues of the rôle of ACh in synaptic transmission and axonal conduction. Obviously this subject cannot be adequately covered. However, an attempt will be made to review the major contributions of studies on the anticholinesterases to the theory of chemical mediation of the nerve impulse.

CLASSIFICATION OF CHOLINESTERASE ENZYMES. In 1926, Loewi and Navratil

(196) discovered an enzyme in the frog heart which was capable of inactivating the "vagus-substance" and ACh. Stedman and co-workers (288), in the course of their studies of the ACh-splitting activity of horse serum, suggested the term "choline-esterase" to describe enzymes of this type. Since then, a massive literature has accumulated on the properties, distribution and differentiating characteristics of the ChE enzymes. A consideration of certain fundamental principles which have emerged from these investigations is necessary for an understanding of the present concept of the mechanism of action of the anti-ChE drugs. Although all controversial points have not been settled and the investigative possibilities of the subject are by no means exhausted, certain facts are now apparent which permit studies of these drugs to be conducted on a sounder basis than previously.

It was noted as early as 1928, by Galehr and Plattner (114), that the ChE splitting activities of serum and whole blood differed, and that at low substrate concentrations the latter was the more active. Therefore, they suggested that the corpuscles were probably primarily responsible for the hydrolysis of ACh in the bloodstream. However, they attributed the activity in this respect to a non-specific surface action rather than to an enzyme. Following extensive studies of the properties of serum ChE of different species by Stedman and co-workers (76, 288) and by Glick (118, 119, 120) and others, Alles and Hawes (5) confirmed the earlier observation of the greater ACh-splitting capacity of erythrocytes at low substrate concentrations, but they considered the action to be due to the presence of a ChE in the red cells. The erythrocyte ChE was further differentiated from that of serum by its ability to hydrolyze acetyl-β-methylcholine, and by its greater sensitivity to changes in pH and salt concentration. Subsequent investigations of the substrate specificities of ACh-splitting enzymes from various sources and their sensitivities to different inhibitors (253) led Mendel and Rudney (216, 217, 218) to propose a general classification of enzymes of this type. Included under the term "pseudo-ChE" were the enzymes from serum, pancreas and other tissues which hydrolyzed ACh, benzoylcholine and several non-choline esters (tributyrin, tripropionin, methyl butyrate) but not acetyl-β-methylcholine, and which exhibited maximal activity in the presence of high concentrations of ACh. The term "true-ChE" was reserved for the enzymes of erythrocytes and nervous tissue which acted only on choline esters, including acetyl-β-methylcholine but not benzoylcholine, and which were inhibited by high concentrations of ACh. As a method for estimating quantitatively the presence of true-ChE, pseudo-ChE and other esterases in tissues, they suggested the determination of the activities against acetyl-β-methylcholine and benzoylcholine, and of the ratio of the rates of hydrolysis of ACh at concentrations of 0.06 and 0.0006 M. It was found that by treating true-ChE with positively charged colloids, such as protamine, the optimal substrate concentration could be raised to a higher level without modification of the substrate specificity. This effect could be reversed by subsequent treatment with a negatively charged colloid (219).

The above classification was criticized by several authors (121, 186), and Nachmansohn and Rothenberg (238, 239) proposed that the enzyme of eryth-

rocytes and nervous tissue be designated "specific-ChE," on the basis of its relative specificity for ACh, to distinguish it from the numerous other ACh-splitting esterases found in serum and various tissues. "Specific-ChE" was characterized as exhibiting a definite pattern of substrate relationships, in that it (a) split ACh at a higher rate than any other substrate, the rates of hydrolysis of other choline esters decreasing with increasing chain length of the acyl group, (b) hydrolyzed acetyl-β-methylcholine, but at a lower rate than ACh, and (c) failed to attack benzolycholine, carbaminoylcholine or esters of simple alcohols. Although they confirmed the inhibition of specific ChE by high concentrations of ACh, they found the optimal level to be considerably higher than that reported by Mendel and Rudney, and attributed the discrepancy to the short periods of determination employed by the previous authors.

The use of specific inhibitors has also been suggested as a method for distinguishing between specific-ChE and related enzymes. Inhibition by neostigmine was one of the criteria used by Easson and Stedman (76) for this purpose, but it has been shown since that this compound is highly potent against nearly all types of ChE. Zeller and Bissegger (322) found that percaine inhibited selectively the ChE of serum, caffeine that of erythrocytes and brain, while morphine was a relatively potent inhibitor of both types. Di-isopropyl fluorophosphate (DFP) has been shown to inhibit the ChE of the serum of most species in concentrations far lower than those required to inhibit erythrocyte or brain ChE (147, 211). Another chemical warfare agent, β-β'-dichlor-diethyl-N-methylamine (DDM) was found by Adams and Thompson (2) to exhibit the reverse type of selective inhibition. These authors proposed the use of the I_{50} (molar concentration producing 50 per cent inhibition) ratio, $I_{50}DDM/I_{50}DFP$, for distinguishing between the two types, the specific-ChEs having relatively low ratios and the non-specific types far higher ratios (*e.g.*, 6.7×10^2 for human brain, 4.0×10^5 for human plasma).

Numerous exceptions to the foregoing generalizations have been reported. Mazur and Bodansky (211) found that mouse brain hydrolyzed triacetin at a higher rate than ACh. This observation may have resulted from the employment of excessively high substrate concentrations. Several marine invertebrates were found by Augustinsson (9) to contain enzymes capable of splitting ACh at high rates and exhibiting little activity towards either acetyl-β-methylcholine or benzoylcholine. He suggested that the ChEs from these sources might be more specific towards ACh than the enzymes previously studied. Hawkins and Mendel (146) reported that planaria and frog brain contain ChEs which are specific according to substrate criteria but which are extremely resistant to inhibition by physostigmine. Furthermore, the enzyme from planaria exhibited normal sensitivity to neostigmine but was not inhibited by high substrate concentrations.

The most satisfactory classification at the present time appears to be the one recently advanced by Nachmansohn and Augustinsson (235). They have applied the term "acetylcholine esterase" to the enzymes of nervous tissue and erythrocytes which catalyze the hydrolysis of ACh at optimal substrate concen-

trations at a greater velocity than any other known substrate, and which fit the general description previously given by Nachmansohn for "specific-ChE." Under the term "cholinesterase" they include both "acetylcholine esterase" and the various enzymes of sera and tissues which promote the hydrolysis of other choline esters, such as benzoylcholine and butyrylcholine, at relatively high rates (237, 264). Other hydrolytic enzymes which manifest specificity for such substrates as atropine (18) and monoacetyl morphine (89) are designated by the general term "esterase."

The pharmacological significance of the foregoing studies rests in the fact that endogenously liberated ACh, at the low concentrations in which it is present in the body, is apparently hydrolyzed exclusively by means of specific-ChE or acetylcholine esterase. Consequently, anti-ChE drugs produce their typical cholinergic effects only when this type of enzyme has been inhibited beyond a certain threshold. This fact was not fully appreciated prior to the studies of the alkyl phosphates, which were shown to be capable of producing practically complete inactivation of serum ChE without causing any significant symptomatology (211). Therefore, most of the earlier studies of the actions of anti-ChE drugs on the serum enzyme provide information only on such phases as absorption, excretion and enzyme kinetics, and do not supply any quantitative data relating enzyme inhibition to pharmacological responses.

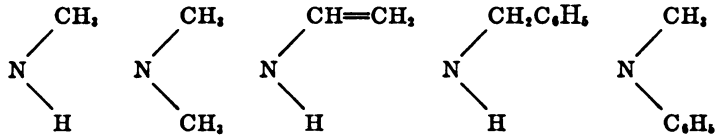
The distribution of the ChE enzymes throughout the animal kingdom and in the various tissues has been reviewed thoroughly in Augustinsson's recent monograph (10).

STRUCTURE-ACTIVITY RELATIONSHIP. *Reversible inhibitors.* The first drug known to inhibit ChE reversibly, physostigmine, has proved to be one of the most potent compounds possessing this action. Early studies designed to elucidate the mechanism of its miotic action led to the synthesis of neostigmine another highly potent anti-ChE. These investigations provide a typical example of the significant modifications of pharmacological activity produced by minor alterations of chemical structure, and reveal the difficulty met in attempting to draw generalizations relating these two factors.

Most of the classical work of Stedman and collaborators and Aeschlimann and Reinert on the relationships between chemical structure and pharmacological activity of the homologues of physostigmine and related compounds was conducted before it was realized that these drugs are potent inhibitors of ChE. After determining the structure of the physostigmine molecule and its inactive degradation product physostigmol (285), Stedman decided that the miotic action of the former was due to the urethane grouping. He then prepared a series of urethanes from dimethylaminophenol (DMP) and hordenine (HO—C_6H_4-*p*-CH_2 CH_2N $(CH_3)_2$) and compared their miotic potencies in cats (282). All three isomers of the dimethylaminophenyl ester of methylcarbamic acid were found to be active, as well as the corresponding *m*-derivative of carbamic acid, the *o*-derivative of ethylcarbamic acid, and the methylcarbamido derivative of hordenine. All phenylcarbamic acid esters were inactive. When the tertiary basic group was converted to a quarternary ammonium group by the synthesis of the corresponding methiodide, a marked change occurred; the activity of the *m*-compounds was intensified, that of the *o*- and *p*-derivatives was abolished. This was interpreted, according to the polarity theory, as an indication that those compounds

which were derived from the most acidic phenol derivatives were the least active. It was noted that the active synthetic compounds and physostigmine have two structural features in common; both are substituted phenyl esters of methylcarbamic acid and both contain a basic group. Subsequently, a series of urethanes was synthesized from isomeric hydroxybenzyldimethylamines (HBDM) and hydroxy-phenylethyldimethylamines (HPEDM) and their miotic properties were investigated (283, 284). Here, too, the methylurethanes were found to be most active, the order of activity for the isomers of HBDM being $o > p > m$. Of the isomers of the methylurethane of HPEDM, the *m*-derivative showed extremely high potency which approached that of physostigmine, and it was given the name miotine. The methyl urethanes of choline iodide and tropeine were also tested and found to be inactive, thus confirming the view that miotic activity was associated only with phenyl esters. Following the announcement by Englehart and Loewi that eserine inhibits ChE (96), the above classes of urethanes were investigated from the standpoint of their ability to inhibit the hydrolysis of methyl butyrate and tributyrin (286). Whereas most of the miotics were highly potent in this respect, the two activities did not show close parallelism. Because of their assumed differences of penetrability *in vivo*, the authors did not consider this discrepancy to rule out the possibility of their producing miosis by esterase inhibition. They suggested that the mechanism of this inhibition probably depended upon the ester structure of the urethanes and their resistance to hydrolysis (287). A detailed pharmacological study was conducted with miotine, the three isomers of the methylcarbamic urethanes of HBDM, and physostigmine. All possessed similar pharmacological properties as revealed by their effects when administered alone or in conjunction with ACh, atropine, nicotine or curare. It was concluded that while the major part of the actions of physostigmine and miotine was dependent upon the inhibition of ChE, an additional factor of direct action on effector organs might be present (315).

Aeschlimann and Reinert (4) studied the structure-activity relationships of 45 additional urethanes. Because of the stability of the dialkyl and diaryl carbamic esters, they concentrated on these types. Eserine-like action was found to be the strongest in the carbamic esters of phenol bases with the following radicals attached to the carbamate nitrogen:



The quaternary salts of the aromatic bases having the nitrogen attached directly to the benzene ring were more active than the hydrochlorides of the corresponding tertiary bases, whereas the reverse was true with compounds having the basic radical in a side-chain. The quaternary salts in general were found to be relatively ineffective when administered orally. Two compounds, the dimethylcarbamic ester of 3-oxyphenyltrimethylammonium methylsulfate (prostigmine or neostigmine) and the corresponding methylphenyl carbamate, were selected as promising drugs.

In addition to the above urethanes, several other quaternary ammonium compounds and tertiary amines are relatively potent inhibitors of ChE.

Methylene blue and other basic dyes having strongly dissociated quaternary ammonium groups are examples of the former type. Conversion of such dyes to tertiary leuko-bases by the addition of alkali results in the loss of their anti-ChE activity. Thionine, which differs from methylene blue in that the methyl radicals in the quaternary ammonium group are replaced by hydrogen atoms, is a considerably weaker inhibitor. This difference was attributed by Massart and Dufait (208) to the increased dissociation of the quaternary group accompanying substitution with alkyl radicals. They demonstrated a parallelism

between dissociation constant and anti-ChE potency with several such dyestuffs. When methylene blue is administered *in vivo*, however, it does not produce the same action as physostigmine (252), apparently because of two factors: the conversion of a large portion of the dye to the inactive leukobase at the pH of the blood, and its combination with the receptors of the effector cells to prevent the access of ACh (250). Although these authors found Congo red and other acid dyestuffs to be relatively inactive as ChE inhibitors, a recent report (166) has suggested that the anticurarizing action of Congo red may be partially due to an anti-ChE effect. The anti-ChE activity of crude curare preparations (213) has been shown to be due largely to impurities, since the purified alkaloids are very weak in this respect (135).

Among tertiary amines having anti-ChE activity, the toad-poison cinobufotenine flavinate, which bears a close structural resemblance to physostigmine, is fairly potent (277). Of several vesicants studied, Thompson (295) found β-β' dichlorodiethyl N-methylamine and trichlorotriethyl amine to be moderately potent ChE-inhibitors *in vitro*, while carbomethoxy-β-chloroethylnitrosamine caused greatest inhibition of the skin ChE when applied ocally. The anti-malarials quinine and atabrine are potent inhibitors (308), whereas paludrine (21), like percaine and related compounds (322), shows little activity against brain ChE but inhibits serum ChE more strongly. Wright's studies of aromatic amino alcohols (319, 320) indicate that anti-ChE and antimalarial activities are unrelated.

There are numerous other anti-ChE drugs in which the amino nitrogen forms part of a heterocyclic ring. The Bernheims (17) noted that morphine and apomorphine in high dilution inhibit brain ChE, and suggested that some of their pharmacological actions might be due to this property. The imidazoline derivatives priscol, privine and otrivin exhibit weak anti-ChE activity but are relatively specific against the different types. Their inhibiting action against the amine oxidases is much greater (266). The action of strychnine (230, 231) has been attributed to its anti-ChE activity but its potency in this respect scarcely justifies such a conclusion in view of the small doses which elicit marked pharmacological effects. Inhibition of ChE is also produced by dilaudid, codeine, desomorphine (321), meperidine (28) and caffeine (242).

The purpose of the present review would not be served by listing all the drugs known to inhibit ChE. Such a table has been assembled by Augustinsson (10), and Bernheim (16) has reviewed the pharmacology of many of the compounds mentioned above.

As stated in the introduction, the fact that a compound is capable of inhibiting ChE does not imply that it produces its pharmacological effects by this mechanism. Moreover, a drug acting primarily as an anti-ChE at one site may act directly on the effector cells at another locus. This phenomenon is illustrated by the studies of Clark and Raventos (55) and Raventos (249, 250) on the pharmacology of some quaternary ammonium salts.

These authors investigated the interactions of homologues of the series $(CH_3)_3NR$, R_4N, $R_nNR'_{(4-n)}$ and $(CH_3)_3N(CH_2)_nC_6H_5$ as well as their effects on the actions of ACh on frog and leech muscle, frog auricle and rat gut. Lower members of the series $(CH_3)_3NR$ had only a curariform action on leech muscle; with the other test objects the lower members produced an ACh-like effect and summated with ACh. The higher members antagonized both the actions of ACh and those of lower members. The remaining series had comparable although somewhat more complex effects. Methylene blue, on the other hand, antagonized the action of ACh on the frog heart and rat gut, had a diaphasic effect on the frog rectus, and potentiated ACh action on leech muscle. On the last named test object, its anti-ChE action apparently predominated, whereas on the frog heart it acted as an antagonist in the same manner as the other quaternary ammonium salts studied.

It would seem likely, therefore, that many of the actions of the anti-ChE drugs, especially the less potent ones, may be due to similar direct effects. The response of a particular effector cell is dependent upon which type of action predominates at that site,—ChE-inhibition, direct effect or cholinergic blockade.

Irreversible inhibitors. During the course of chemical warfare research it was established that the series of esters of fluorophosphoric acid inhibited ChE. Of the series, the di-isopropyl derivative was the most active. Also during the war years Bloch and Hottinger (22, 154) demonstrated that tri-orthocresyl phosphate was a potent anti-ChE (see below). At the close of the war the Technical Intelligence Committee investigating research progress in Germany learned that the Germans had been employing hexaethyltetraphosphate (HETP) as an insecticide. DuBois and Mangun (72) soon demonstrated that this alkyl phosphate was capable of inhibiting ACh-splitting enzymes. Although only few studies have been published on the structure-activity relationship of the alkyl phosphates relative to the inactivation of ChE, the outstanding contribution of Brauer (27) has provided much information in this field as well as basic facts pertaining to the reaction between enzyme and inhibitor (see below).

Brauer studied a group of 16 phosphate esters. He used as his source of enzyme the cholinesterase of fractionated human plasma (fraction IV-6). Highly active compounds were found among all types of phosphates including polyphosphates, phosphophosphines, thiophosphates and sulfonephosphates. Although the number of compounds studied was too small to permit detailed analysis of the effects of structure on the degree of anti-ChE activity, and the exact constitution of certain of the alkyl phosphates is not known, nevertheless Brauer was able to draw fundamental conclusions concerning the structure necessary for inhibition of ChE. Thus all the active compounds had in common the grouping P—O—R where R may be an alkyl or an aryl radical. However, the presence of this grouping alone is not a sufficient condition for activity. For example, in the following pairs of compounds the first member is inactive, the second active: (1) triethyl phosphate, tetraethylpyrophosphate; (2) trimethyl phosphate, dimethyl fluorophosphate; (3) tri-*p*-cresyl phosphate, tri-*o*-cresyl phosphate. Brauer points out that in each of the above pairs, the active compound contains an arrangement which would be expected to have a high free-energy content as follows: (1) the pyrophosphate linkage; (2) the analogous anhydride of hydrofluoric acid dimethyl phosphoric acid and (3) the stearic strain which probably exists in tri-*o*-cresyl phosphate. Of the various compounds studied by Brauer, HETP and tetraethylpyrophosphate (TEPP) exhibited the highest activity and produced 50 per cent inhibition of the enzyme when employed in the molar concentrations of 1.0×10^{-9} and 8.6×10^{-10}, respectively.

DYNAMICS IN VITRO. *Reversible inhibitors.* In an earlier section it was pointed out that the hydrolysis of ACh by the non-specific enzymes of serum and various tissues is characterized by a direct relationship between reaction velocity and substrate concentration. This type of relationship is expressed mathematically by the well-known Michaelis-Menten formulation (222), according to

which a single molecule of substrate combines with one enzyme center to form a complex; the breakdown of the combination into the reaction products and the free enzyme proceeds as a monomolecular reaction. Other substances which can combine with the enzyme but which are not necessarily split by it act as inhibitors, the potencies of which are proportional to their affinities for the enzyme compared with that of the substrate. Matthes (209) employed these principles in one of the earliest quantitative studies of the hydrolysis of ACh by blood and serum and the inhibitory effect of physostigmine. He assumed that the inhibition was a non-competitive monomolecular reaction and demonstrated its reversibility by dialysis.

The inhibition of hydrolysis by an excess of substrate, as observed with the specific ChEs of nervous tissue and erythrocytes, represents a type of reaction that was studied by Haldane (134). In such cases, when reaction velocity is plotted against the logarithm of the substrate concentration, a bell-shaped curve is obtained, the peak of which occurs at the optimal substrate concentration. According to Haldane's interpretation, in order to bring about reaction, enzymes of this type and substrate molecules must combine at two spots, forming the complex E = S. In the presence of excess substrate, an inactive type of combination tends to occur, $E\begin{smallmatrix}S\\S\end{smallmatrix}$, which competes with the formation of the reactive complex. For specific ChE, it has been suggested that the two points of combination are represented by a group on the enzyme molecule which combines with the ester linkage of the substrate, and an appropriately located negatively charged group which combines with the positively charged quaternary nitrogen of ACh (9, 322). Combination between ACh and non-specific esterases, where the Michaelis-Menten formulation is applicable, is assumed to occur at only one locus (9). This concept also provides a possible explanation for the shifting of the optimal substrate level of specific ChEs by treatment with positively charged protamines, which presumably results in blocking of the negative groups of the enzyme molecules.

In an investigation of a group of enzymes which acted in accordance with the Haldane theory, Lineweaver and Burke (105) developed graphical procedures for determining the dissociation constants of the different complexes formed by the enzyme, and for distinguishing between competitive and non-competitive inhibition. This distinction was based on the assumption that with the former type the degree of inhibition is decreased with increasing concentrations of substrate in the presence of a constant amount of inhibitor, whereas when inhibition is non-competitive it is not influenced by substrate concentration. Applying this method to an investigation of the inhibition of a purified horse serum ChE by several compounds, Roepke (257) obtained results which indicated that inhibition was produced competitively by choline, carbaminoyl choline, arsenocholine, acetyl-β-methylcholine, tetramethylammonium chloride

atropine and several other substances, but non-competitively by physostigmine and neostigmine. He noted, however, that in the presence of the last two drugs, rates of hydrolysis progressively increased during the determination periods, which indicated that equilibrium had not been attained between enzyme, inhibitor and substrate, and he suggested that this factor might have distorted his results. He also calculated the dissociation constants for the enzyme-inhibitor complexes by means of the Michaelis-Menten equations. Using modifications of the same types of mathematical treatments, Eadie (74) arrived at entirely different conclusions, namely, that the inhibition of dog serum ChE by physostigmine or neostigmine is competitive and that equilibrium is reached within a few minutes after mixing. On the assumption that two molecules of inhibitor combine with one of enzyme, he obtained extremely low dissociation constants.

A major departure from the foregoing methods of treating reversible enzyme-inhibitor combinations was made by Easson and Stedman (75) in a study of the kinetics of the system horse serum ChE-ACh-inhibitor (physostigmine or neostigmine). Instead of treating the reaction as a first order one, as previous workers had done on the assumption that the enzyme centers were present in infinitesimal concentration, they regarded it as truly bimolecular. This concept was greatly expanded and placed on a sound quantitative basis by Straus and Goldstein (293). They introduced the term "specific concentration," defined as the ratio of the molar concentration of enzyme or inhibitor to the dissociation constant of the complex formed. As determined by the *specific concentrations* of enzyme in a given system, three "zones of behavior" were described, characterized by significant differences in functions relating the concentrations of the components. In zone A, where the specific concentration of the enzyme is small and practically all the inhibitor exists in the free state, inhibition was shown to be a function of only the specific concentration of the inhibitor. In such cases, the classical equations assuming a pseudomonomolecular reaction were shown to be applicable. In zones B and C, however, where the specific concentrations of enzyme become increasingly greater, the reactions between enzyme and inhibitor must be treated as bimolecular and stoichiometric, respectively. Equational and graphical methods were presented for determining zone boundaries, approximate specific enzyme concentrations and the zone in which a given system is operating, and for treating reactions in each zone. The application of the method was demonstrated with the system horse serum ChE-physostigmine. The change in inhibition resulting from dilution of a system was stressed. Furthermore, it was suggested that at certain locations in the body where enzymes are highly concentrated, such as the neuromuscular junction, zone C relationships might operate. Consequently all potent reversible inhibitors would be approximately equally effective at such sites even though their individual potencies differed within wide limits. Goldstein (123) extended the study to include the competitive effect of the substrate in the system ChE-ACh-physostigmine. The same three zones of behavior were found to occur under this condition, and it was shown that the zonal phenomenon could be utilized for determining the number of molecules of substrate or inhibitor combining reversibly with a single enzyme

center. In contradiction to Eadie's conclusion, this number was found to be one in the system under study, and combination between ChE and physostigmine in moderate concentrations was shown to proceed slowly, as Roepke had observed. In addition, methods were developed to correct for such factors as dilution, displacement of inhibitor by substrate and vice versa, and for determining the rate of destruction of the inhibitor. These investigators have placed the study of reversible enzyme-inhibitor systems on a far more satisfactory quantitative basis, and have explained many of the earlier discrepancies mentioned above. From a practical viewpoint, most systems studied operate in zone A, and, as the authors point out, can be treated by the older methods provided the other factors mentioned are properly taken into consideration. Thus, in Augustinsson's (10) recent investigations, in which he utilized only relatively dilute ChE systems *in vitro*, the equations of Michaelis and Menten, Haldane, and Lineweaver and Burke were found to be quite satisfactory for treating his data. However, practically all *in vivo* studies of the degree of inhibition of a given enzyme produced by administration of a reversibly acting drug necessitate the application of Strauss and Goldstein's correction factors to avoid gross errors. For the mathematical treatment of the above theories, reviews (10, 23) and the original papers should be consulted.

Goldstein (124) has recently reported on the types of inhibition of a highly purified human plasma ChE produced by several compounds. The group of reversible but non-competitive inhibitors included methylene blue, acriflavine, morphine, atropine, strychnine, amphetamine, phenobarbital, sulfanilamide, procaine, choline and acetyl-β-methylcholine, all of which showed a 1:1 ratio for molecules of inhibitior to active centers. Physostigmine, neostigmine and carbaminoylcholine inhibited reversibly and competitively. As noted below, these three compounds were the only ones found by Koelle (169) to offer a high degree of protection for brain ChE against irreversible inactivation by DFP.

Ellis and coworkers (90, 92, 93) have studied the kinetics of the destruction of physostigmine in some detail and have investigated the properties of the breakdown products. The non-enzymatic decomposition of physostigmine in buffered solutions was found to be a bimolecular reaction, the rate of which was dependent upon the concentrations of physostigmine and OH^-. In the presence of ChE, the velocity of destruction was also related to the concentration of the enzyme, provided it was not inhibited beyond about 80 per cent of its normal activity. Physostigmine was converted to eseroline by hydrolytic cleavage of the carbamate group, and was then oxidized to the quinoid rubreserine. This was converted to eserine blue, a compound of undetermined structure, which was further oxidized to eserine brown. Rubreserine and eserine blue were shown to have strong anti-ChE activity (about 1/100th that of physostigmine) and showed comparable pharmacological effects, whereas eseroline and eserine brown were devoid of this property.

Irreversible inhibitors. It is obvious from the above discussion that the kinetics of the inhibition of ChE by reversible inhibitors are so complex, even under controlled conditions *in vitro*, that quantitative studies relating enzyme inhi-

bition to pharmacological actions *in vivo* are impractical if not impossible. The discovery of the irreversible anti-ChEs has provided the pharmacologist with a research tool that can be used much more effectively than the reversible inhibitors to elucidate the funamental rôle of ACh and ChE in various physiological processes. The heuristic value of these drugs is evident in the extensive literature that has already accumulated. However, even in the case of irreversible anti-ChEs the interpretation of experiments *in vivo* must rest upon a fundamental understanding of the dynamics of the reactions between ChE and irreversible inhibitors *in vitro*, as well as a knowledge of the reactions of the inhibitors with substances other than ChE.

The irreversible anti-ChEs were first studied during the course of chemical warfare research. It was only at the termination of the war that security regulations permitted publication in the open literature. Consequently it is difficult to follow the chronological sequence of events in the development of this field from the open literature.

The first studies of the pharmacological actions of the alkyl phosphates apparently were conducted by Adrian, Feldberg and Kilby (3). They immediately deduced that the prolonged miosis that followed exposure to DFP could be explained more readily by ChE inhibition than by a direct action of DFP on effector cells. They then demonstrated the high activity of DFP in inhibiting ChE. Subsequently Mackworth and Webb (202) first suggested that the inactivation of ChE by DFP was irreversible since the activity of the enzyme could not be restored by dialysis. By an odd coincidence Bloch and Hottinger (22, 154) were conducting at the same time independent investigations on tri-*o*-cresyl phosphate. They observed that the compound irreversibly inhibits ChE and their papers provide the first published reports on ChE inhibitors of the alkyl phosphate type.

The extensive investigations of Mazur and Bodansky (211) further characterized the nature of the reaction between ChE and DFP. They demonstrated that the ChEs of different species and tissues varied in susceptibility to inhibition. In general, the enzyme in the sera of most species, the rabbit representing an exception, was more readily inhibited than that from red cells or nervous tissue. They were unable to reactivate the enzyme by dialysis, dilution or treatment with phosphatase, and concluded that the inhibition was irreversible.

More intimate details of the nature of the reaction between ChE and alkyl phosphate inhibitors have been provided by Brauer (27). He worked for the most part with HETP and TEPP, but his observations may apply to the entire group of alkyl phosphates which inhibit ChE. Brauer employed red blood cells and fractionated plasma as his source of enzymes. He observed that the relation between concentration of inhibitor and degree of enzyme inhibition was linear over a wide range. Such a stoichiometric relation between the number of active enzyme centers destroyed and the number of inhibitor molecules present would be anticipated in a reaction involving irreversible inhibition. Brauer further demonstrated that in the reaction between alkyl phosphate and ChE, the inhibitor as well as the enzyme is destroyed. Furthermore the resulting complex

formed contains no phosphorus. Brauer has proposed the following reactions between enzyme and inhibitor which are compatible with his observations:

$$\equiv POR + EH \longrightarrow \equiv POH + ER$$
$$\equiv PX + EOH \longrightarrow \equiv POH + EX$$

where $\equiv$ POR or $\equiv$ PX is the inhibitor, EH or EOH the active enzyme, ER or EX the inactivated enzyme, R an alkyl or an aryl group, and X a halide.

The alkyl phosphates show rather marked specificity in their chemical reactions when this is measured in terms of loss of anti-ChE activity (27). For example, anti-ChE activity is not lost in the presence of various amino acids, ethanol or phenol. In fact ChE denatured with ethanol, acid or heat loses its ability to react with these inhibitors. Crystalline human albumin and human fibrinogen also fail to react. However, fractions of human plasma containing high globulin concentrations show some ability to inactivate TEPP, although less than the main esterase-bearing fractions.

There is presumptive evidence that physostigmine, neostigmine and carbaminoylcholine react reversibly with the same moiety of the ChE molecule as do DFP and TEPP irreversibly. As will be discussed below, the prophylactic administration of certain reversible inhibitors of ChE protects animals from the effects of DFP (175). To explain this phenomenon Koelle (169) first reacted brain ChE with various reversible inhibitors, then added DFP and after a suitable interval dialyzed the mixture. In the presence of any of the above three compounds, in contrast to numerous other inhibitors, the ChE was protected from inactivation by DFP. The possible significance of this finding relative to competitive and non-competitive reversible inhibitors of ChE has already been discussed.

Nachmansohn and coworkers have also studied the kinetics of the reaction between ChE and DFP (240, 241). They used as their enzyme a highly purified ChE obtained from the electric tissue of *Electrophorus electricus* as well as ChEs from other sources. A stoichiometric basis of the reaction between ChE and inhibitor was observed. The reaction between enzyme and DFP was found to depend upon the concentration of enzyme as well as that of inhibitor. The greater the dilution of the enzyme, the higher is the excess of DFP required for inactivation so that at the low enzyme concentration generally used for manometric determination a ratio of 100,000 molecules of DFP to one of enzyme is required for 50 per cent inhibition. This ratio is reduced to 25 to one at higher enzyme concentrations.

Nachmansohn's group has presented evidence to show that whereas DFP inhibits cholinesterase immediately, irreversible inactivation is a function of time, temperature and concentration of inhibitor. For a period of a few hours at low temperatures and low inhibitor concentrations, the enzyme-inhibitor complex can be largely dissociated with reactivation of the enzyme. As the temperature and concentration of inhibitor are raised, this period becomes progressively shorter. Nachmansohn postulates that a loose addition complex is first formed between enzyme and inhibitor, following which a chemical reaction which cannot

be easily reversed develops between the active group of the enzyme and the DFP molecule. The evidence which Nachmansohn and his associates marshal to support the theory of the rôle of ACh in axonal conduction (see below) depends almost entirely on the concept of an early reversible phase of the inactivation of ChE by DFP.

A discussion of the reactions of the alkyl phosphates *in vitro* would not be complete without mention of the susceptibility of the compounds to inactivation by hydrolysis. Most if not all members of the series are unstable in aqueous solution. However, their half-life is sufficiently long to permit most experimental procedures. Solutions in anhydrous oily solvents are stable. In fact a solution of DFP in peanut oil can be autoclaved or kept at room temperature for several months without loss of activity (170). Finally Mazur (210) has demonstrated that an enzyme present in liver and other tissues (phosphofluorase) is capable of inactivating DFP by breaking the bond between fluorine and phosphorus.

Reactions in Vivo. The failure of earlier workers to take into account the differences between the ChEs of the plasma and various tissues, or the kinetic factors involved in reversible inhibition resulted in conclusions which the data obtained did not vindicate. Thus, Manning, Lang and Hall (203) attributed a dual action to physostigmine,—ChE-inhibition with preservation of endogenous ACh and a direct stimulating action on effector cells,—because they were able to obtain responses by injecting additional doses of the drug after it had been given in amounts sufficient to produce maximal inhibition of serum ChE. Heymans *et al.* (149) more recently arrived at a similar conclusion regarding the actions of physostigmine, neostigmine and DFP, although they apparently did not determine directly the inhibition of tissue ChE. The quantitative studies of Clark and Raventos (56) on the kinetics of ACh-hydrolysis *in vivo* would be worth repeating in the light of the facts known at present.

Krayer, Goldstein and Plachte (176) investigated the inhibition of serum ChE and rate of disappearance of physostigmine from the bloodstream when the drug was given by single injections and continuous intravenous infusions to dogs. They applied the necessary correction factors for equilibrium, destruction of inhibitor, dilution and competition discussed above (123, 293). A given level of inhibition could be maintained indefinitely by intravenous infusion at a predetermined rate; when the infusion was stopped, serum ChE activity rose rapidly to normal regardless of how long it had been depressed. Fasciculation of skeletal muscles occurred when the level was below 15 per cent of normal. The rate of destruction of physostigmine by the organism, in which the kidneys appeared to play a small part, was found to increase with increasing concentrations of the drug, so that after the ChE activity of the serum was depressed below 30 per cent of normal, it was necessary to give increasingly larger amounts to produce further increments of inhibition. Similar results have been obtained by Root (258). with neostigmine in dogs and in patients with myasthenia gravis. Both the liver and kidneys of dogs were found to take part in the destruction of the inhibitor.

DuBois and associates (71) have demonstrated the importance of penetration in governing the site of action of an anti-ChE drug. Three compounds were found to cause approximately equal inhibition of rat brain and submaxillary ChE *in vitro* (carbamic acid, N,N-dimethyl-4 dimethylamino-3 isopropyl phenyl ester methiodide, physostigmine and neostigmine). When minimal lethal does were injected subcutaneously, the inhibition of ChE from these two sources and from the serum showed marked differences.

DFP reacts with ChE *in vivo* to produce irreversible inactivation of the enzyme. The reaction presumably is similar to that which occurs *in vitro*. However, certain of the other alkyl phosphates apparently react somewhat differently *in vivo* than *in vitro* and these discrepancies will be discussed.

In early experiments in which human subjects were exposed to low concentrations of DFP, Mazur and Bodansky (211) observed that serum ChE was completely inactivated at a time when the subjects showed little or no response to the drug. This surprising finding was soon explained by studies which defined the relative susceptibility to inhibition by DFP of ChEs of different tissues. In general, it has been shown by several investigators that the acetylcholine esterase of nervous tissue and erythrocytes is less susceptible to inhibition by DFP both *in vitro* and *in vivo* than the ChE of plasma (2, 145, 147, 170).

DFP inactivates ChE *in vivo* with extreme rapidity. Thus death can occur within a few minutes after the intravenous injection of a dose capable of inactivating most of the tissue ChE. The signs and symptoms of acute and chronic poisoning are discussed below. The lethal dose of DFP in the monkey is approximately 0.2 mgm per kg. At the time of death the ChE activity of the brain is virtually zero (211). Assuming that DFP is distributed throughout the total body water, the concentration of anti-ChE in the body fluids at equilibrium would be approximately 1.7×10^{-6} M. The concentration of DFP required to produce 50 per cent inactivation of the ChE of monkey brain *in vitro* is 3.2×10^{-6} M. Moreover, the concentration of DFP required to inhibit horse serum ChE is the same for the native serum and a purified enzyme preparation from the same source. Likewise, if human brain extract is heated to destroy ChE activity, the addition of this extract to human serum does not affect the sensitivity of the serum ChE to inhibition by DFP. All these facts attest to the rather marked specificity of the reaction between DFP and ChE, both *in vivo* and *in vitro*. However, there is ample evidence that DFP can react both *in vivo* and *in vitro* with substances other than ChE. Evidence for a reaction between DFP and globulin was presented above. The lethal dose of DFP is much higher when the inhibitor is injected into the portal circulation than when introduced into the systemic circulation (150). This may be due to an enzymatic inactivation of the alkyl phosphate or possibly to a reaction between DFP and substances other than ChE. Also Harvey and coworkers (140) have shown that when DFP is injected into the brachial artery of human subjects and is excluded from the general circulation for a brief period by venous occlusion, no systemic effects of DFP are evident upon release of the occluding tourniquet. Inasmuch as the doses employed were large (2.0 mgm) it must be presumed that the major

portion of the DFP reacted with and was inactivated by substances other than cholinesterase. When neostigmine was administered in the same manner there was no evidence of localized destruction, and systemic as well as local effects were observed.

In vitro evidence that neostigmine and DFP react with the same moiety of the ChE molecule and that the presence of neostigmine protects the enzyme from irreversible inhibition by DFP has been presented above. The same antagonism can be demonstrated *in vivo* and has practical significance in therapy. Koster (175) first demonstrated that the prophylactic administration of physostigmine protected animals from the effects of DFP. Harvey and associates (140) showed in humans with myasthenia gravis that the injection of DFP into the brachial artery improved muscle strength in the treated arm for days. Neostigmine produced a similar response, but only for a few hours. If DFP was administered after neostigmine, the response in no way differed from that to neostigmine alone. Apparently the presence of neostigmine protects ChE from irreversible inactivation by DFP *in vivo* as well as *in vitro*. This finding is of great practical importance. In the treatment of glaucoma, myasthenia gravis and ileus, the alkyl phosphates are often employed only after the reversible ChE inhibitors have proved relatively ineffective. If an alkyl phosphate is to be given to a patient who has already received neostigmine or physostigmine, its administration should be delayed until the reversible inhibitor has been excreted.

The fact that DFP inhibits ChE irreversibly *in vivo* has provided the means for studying the rate of resynthesis of this important enzyme. Plasma cholinesterase apparently is formed in the liver and is resynthesized within a few weeks (130, 170, 211). The rate of resynthesis is appreciably diminished as a result of liver damage (130, 314). The ChE of brain and muscle is replaced much more slowly and as long as three months may be required after inactivation with DFP before normal ChE activity returns (170, 211). The curve depicting regeneration rates is parabolic, with 50 per cent recovery occurring within the first few weeks. Erythrocytes are apparently incapable of resynthesizing ChE and values for red cell ChE activity return to normal after the inactivated cells have been replaced in the course of their physiological destruction. Thus the rate of return of erythrocyte ChE activity provides information on the life cycle of the red cell (130, 170).

All the alkyl phosphates thus far studied irreversibly inactivate ChE *in vitro* and attempts to reactivate the enzyme have been uniformly unsuccessful. However the pharmacological responses which follow the administration of HETP or TEPP are much more evanescent than those elicited by DFP. For example, topical application of DFP to the eye produces a miosis which may last for days or weeks whereas the effect of HETP is gone within 12 to 24 hours (69). There is evidence of a preliminary nature that the regeneration of tissue ChE occurs much more rapidly following the administration of HETP or TEPP than following DFP, especially during the initial phase (69, 127). It has been postulated therefore, that HETP *in vivo* forms a more labile combination with ChE than does DFP, and a significant amount of ChE can be reactivated and thereby foreshorten pharmacological responses.

AUTONOMIC EFFECTOR CELLS. The classical experiments of Loewi and his associates have resulted in the general acceptance of the hypothesis that stimulation of autonomic nerves results in the liberation of chemical substances at their endings, the so-called chemical mediators of the nerve impulse. The designation autonomic nerves as either cholinergic or andrenergic logically followed the demonstration of the nature of the chemical mediators. Further understanding of the mechanism by which stimulation of cholinergic nerves elicited discrete responses in effector cells was provided by studies of the properties and distribution of enzymes capable of hydrolyzing ACh. With this background, the classical observation of Englehart and Loewi (96) that physostigmine inhibits ChE affords an adequate explanation of the complex parasympathomimetic actions of the alkaloid and provides one of the rare instances in which the basic mechanism of action of a drug has been elucidated.

Studies of the effects of physostigmine on autonomic effector cells have proved invaluable in establishing present physiological concepts of autonomic function. Moreover the drug has been an essential tool in helping to establish firmly the incontrovertible rôle of ACh as the chemical mediator released from postganglionic cholinergic fibers. This has resulted in a large and familiar literature on the pharmacological actions of physostigmine and related compounds on autonomic effector cells which cannot profitably be reviewed at this time. Suffice it to say that there are few if any actions of physostigmine on smooth muscles and exocrine glands which cannot be explained on the basis of ChE inhibition and the consequent enhancement of the effects of endogenous ACh. Thus the response of an effector organ to physostigmine is largely conditioned by the activity of the cholinergic nerves which it receives. It also follows that physostigmine will exert no prominent actions on autonomic effector cells which have been deprived of cholinergic innervation.

Since the discovery of the irreversible anti-ChE activity of the alkyl phosphate compounds, these concepts have been confirmed and in no way basically altered. The actions of the alkyl phosphates on autonomic effector cells are those that would be anticipated of an irreversible inhibitor of ChE. The responses are similar to those obtained following the administration of physostigmine or neostigmine but they are much more prolonged. These will be briefly reviewed inasmuch as the literature is rather recent.

Eye. The actions of the alkyl phosphates on the eye have been investigated more extensively than those on other organs (192, 268, 269, 306). Following exposure to the vapors of the alkyl phosphates or topical application of solutions, an intense miosis develops within a few minutes. This is followed shortly by spasm of accommodation with the lens fixed for near vision. Following a single instillation of DFP in normal human eyes the spasm of accommodation lasts for days and the miosis persists for weeks. On the other hand, the effects of HETP are much less persistent (69). The possible explanation of the evanescent action of HETP has been discussed above.

The mechanism of the miotic action of DFP has been investigated by Leopold and Comroe (192). They demonstrated that the chronically denervated pupil does not constrict following the topical application of DFP. Thus DFP, like

physostigmine (7) and neostigmine (190), has no direct action on the sphincter muscle cells of the iris and exerts no pharmacological effect on the absence of a source of ACh.

A decrease in intraocular tension accompanies the miotic action of DFP. In the rabbit this is invariably preceded by an initial rise in tension, a phenomenon which is occasionally seen in glaucomatous eyes. Von Sallmann and Dillon (306) have shown convincingly that the rise in tension results from the vasodilatation produced by the preserved ACh. As a result of arteriolar dilatation the ciliary capillaries passively dilate and become more permeable to protein which gains access to the aqueous. The rise in tension can be prevented by vasoconstrictors.

The miosis and spasm of accommodation produced by DFP can be overcome by high concentrations of atropine. The weaker parasympatholytic agents and the sympathomimetic drugs are much less effective. Conversely, high concentrations of DFP (0.2 per cent) effectively antagonize an atropine-induced mydriasis.

Following the systemic administration of DFP the actions on the eye are not prominent. Therapeutic doses in humans cause few signs or symptoms referable to the eye, and miosis is prominent in experimental animals only when lethal doses are administered.

Lung. DFP, physostigmine and neostigmine cause constriction of bronchial muscle (126) and increased secretion of bronchial glands. These effects of the alkyl phosphates are very prominent in animals receiving large doses and contribute significantly to the lethal effects of this group of compounds. The actions on the lung are also apparent in humans breathing the vapors of alkyl phosphates. Following the systemic administration of therapeutic doses there may be a feeling of substernal tightness which may be due largely to cardiospasm.

Gastrointestinal tract. The isolated intestines of the rabbit and cat have been found to respond to DFP in the same manner as to physostigmine (11). After prolonged immersion in either drug the preparations continued to contract spontaneously but were not affected by further doses of the same or the other agent. Inglefinger (160) has reviewed the earlier literature on the action of drugs on intestinal motility. The actions of DFP on the gastrointestinal tract *in vivo* are prominent. In animals there is an increase in tone of the intestinal muscle as well as in the rate and amplitude of contraction (228). In the human, the intramuscular injection of one to three mgm of DFP causes a marked increase in the motility of the small and large intestine (129). This overactivity subsides within three to six hours despite the fact that ChE presumably is irreversibly inhibited. However, the bowel remains hypersensitive to other stimulants such as morphine, posterior pituitary extract and neostigmine for as long as one to three weeks. For example, doses of neostigmine or pitressin which normally are without effect produce abdominal cramps, nausea and frequently vomiting and diarrhea in a DFP-sensitized individual. The intestinal actions of DFP can be antagonized by atropine and meperidine. Morphine also reduces the motility but increases the tone.

The actions of DFP on the bowel provide the basis for its use in the treatment

of abdominal distention. However, they seriously interfere with other therapuetic applications of the drug in that patients may experience epigastric distress, abdominal cramps, nausea, vomiting and diarrhea. Atropine is of limited value in alleviating these symptoms.

Cardiovascular system. The replacement of the pericardial fluid of the turtle heart with 0.0004 M DFP had no direct effect on the normal rhythm but greatly increased the degree of inhibition produced by vagal stimulation (58). In the isolated rabbit heart, large doses of DFP produced a brief period of inhibition, an action apparently unrelated to its anti-ChE effect. Subsequently, the preparation exhibited marked and irreversible sensitivity to inhibition by ACh (248). Neither therapeutic doses of the alkyl phosphates in humans nor small doses in animals have prominent effects on the cardiovascular system. Presumably the peripheral effects of ACh are balanced by nicotinic actions on the adrenal medulla and sympathetic ganglia. With large doses the blood pressure progressively falls to shock levels. Complete A-V block occurs. The cardiovascular effects can be prevented by atropine (69, 228). The actions of neostigmine on the cardiovascular system have been studied in detail by Mendez and Ravin (220), and its electrocardiographic effects have been analyzed by Goldfinger and Wosika (122).

Miscellaneous. The effects of the alkyl phosphates on other autonomic effector cells have not been studied in detail but certain observations have been made in the course of studies of the general systemic actions of this group of anti-ChEs. DFP (26) and physostigmine (271) enhance secretion by the submaxillary gland. The sweat glands are stimulated by DFP. Thus 20 per cent of patients receiving DFP may perspire excessively (130). Less frequently lacrimation and salivation may be observed. However, increased secretion of these glands, especially the salivary, is prominent in animals receiving lethal doses. In the patients of Harvey and associates who received injections of DFP in the brachial artery, increased sweating occurred in the arm below the point of injection and persisted for days (140). The actions of DFP on the bladder are not prominent in patients receiving therapeutic doses. Urinary frequency is noted occasionally. Dogs receiving DFP over long periods exhibit urinary incontinence (171).

In summary, it may be said that nearly all the actions on the autonomic effector cells of the drugs discussed above can be accounted for by their inhibition of tissue ChE when they are given in moderate doses. High concentrations applied to isolated organs have many effects which are undoubtedly direct ones, but it is questionable that these are obtained *in vivo* following systemic administration.

AUTONOMIC GANGLIA. The theory of the chemical mediation of the nerve impulse, first proposed to explain the transmission of impulses from postganglionic nerves to effector cells, was next extended to include synaptic transmission in autonomic ganglia. Thus, preganglionic fibers synapsing with either cholinergic or adrenergic postganglionic neurons were designated as cholinergic. The theory of chemical mediation at the ganglionic synapse has not received the general acceptance accorded the chemical transmission of postganglionic impusles

to autonomic effector cells. However, it explains many of the actions of anti-ChEs at a ganglionic site. It is not within the scope of the present review to consider this controversial field in its entirety. Attention will be focused on those investigations which help to elucidate the pharmacological actions of the anti-ChE drugs on autonomic ganglia and on the contributions that these drugs have made to an understanding of the physiological events associated with ganglionic transmission.

In 1933, Kibjakow (167) reported that when the preganglionic fibers of the artificially perfused cervical ganglion of the cat were stimulated, the effluent contained a substance which when injected into the perfusate flowing to another ganglion resulted in contraction of the nictitating membrame. The substance was collected in eserinized perfusate and identified pharmacologically as ACh by Feldberg and Gaddum (99). They found that the addition of ACh to the inflowing perfusate elicited a similar action which could be potentiated by physostigmine. These results were interpreted to indicate that ACh normally acts as the transmitter of impulses from the preganglionic fibers to the ganglion cells. The theory was extended to include the transmission of splanchnic nerve impulses to the cells of the adrenal medulla by the confirmation of the earlier observation of Stewart and Rogoff (289) that the administration of physostigmine greatly augmented the output of epinephrine when the nerves to the gland were stimulated, and by the additional finding that ACh was liberated from the adrenals during such stimulation (100). Further studies by Feldberg and Vartiainen (101) showed that whereas small doses of physostigmine potentiated responses to preganglionic stimulation, larger doses had a paralytic effect, like that of nicotine, without interfering with the liberation of ACh. No ACh was liberated in the ganglion following antidromic stimulation of the postganglionic fibers. These results were confirmed by MacIntosh and coworkers (144, 165, 200) who also found that the inclusion of calcium, oxygen and glucose or some other metabolite in the perfusion fluid was necessary for the prolonged continuation of both the discharge of ACh and the transmission of the excitatory effect following preganglionic stimulation. The stimulating effects of potassium on the ganglion were attributed by Brown and Feldberg (36) to the liberation of ACh by the ion, while ganglionic inhibition by high concentrations of calcium was considered the result of its antagonizing this action of potassium.

The foregoing data were presented by their authors as additional support to the neurohumoral theory. However, Eccles (78) raised objections to the hypothesis on the basis of the extremely rapid decay of the synaptic transmitter (1.5–2.5 m sec) which he did not believe could result from the enzymatic hydrolysis of a chemical mediator. Furthermore, he had noted previously (77) that the administration of moderate doses of physostigmine had no effect on ganglionic potential waves or on the facilitation curve of the postganglionic fibers; large doses produced only reduction or abolition of ganglionic potentials. Rosenblueth and Simeone (261, 262) reinvestigated these aspects of the problem and concluded that the ACh theory provided a more satisfactory basis than the electrical theory for interpreting most of their observations, including (1) the long

synaptic delays of some ganglionic cells, (2) the lack of correlation between synaptic delay and facilitation or inhibition, (3) the lack of correlation between after-potentials and responsiveness of ganglion cells and (4) repetitive responses to single nerve volleys. Physostigmine or neostigmine was found to slow the rate of decline of the ganglionic mediator; Eccles' negative results in this respect were attributed to his having used insufficient doses of physostigmine. In the light of his more recent findings, Eccles (79) has favored a dualistic theory of ganglionic transmission, with the major emphasis on the electrical component. Thus, he found the characteristic synaptic potentials set up by single or double preganglionic volleys to be unaltered by physostigmine, and considered that they resulted from the depolarization of the ganglion cell by the direct effect of the action currents of the preganglionic fibers. With tetanic stimulation of the preganglionic fibers, however, physostigmine decreased the stimulation frequency necessary to obtain summation and produced a prolongation of after-discharge associated with the appearance of a prolonged potential superimposed upon the normal synaptic potential. The prolonged potential was attributed to the effect of accumulated ACh which was presumably liberated normally in amounts too small to have any significant rôle in synaptic transmission. For further amplification of this viewpoint, the reader is referred to the recent reviews by Eccles (82, 84).

Arguments against the theory of the transmission of ganglionic impulses by ACh have also been advanced by Lorente de Nó (197, 198). When he repeated the perfusion experiments of Kibjakow (167) he obtained no direct correlation between preganglionic stimulation and ACh liberation, but noted that the latter was usually associated with damage to the cells, as revealed by histological examination. In keeping with these results were the findings of others that the amounts of ACh released from eserinized ganglia with natural circulation were extremely small (99, 200). It was suggested by MacIntosh that this might indicate a rapid resynthesis of ACh under physiological conditions to a precursor substance by some physostigmine-resistant mechanism (201). Recently, Emmelin and MacIntosh (95) have pointed out that the earlier perfusion studies were conducted with an eserinzied Lock's solution of a high pH (about 8.5) which caused the tissues to become edematous within a short time. Using a modified perfusion fluid of pH 7.4, heparinized plasma or defibrinated blood containing adequate amounts of an anti-ChE agent (physostigmine, DFP or TEPP), they consistently obtained a constant amount of ACh in the venous effluent following each preganglionic volley.

Certain resemblances between the neuromuscular junction and autonomic ganglionic synapses have been noted (50). Just as motor denervation renders the former more sensitive to injected ACh, preganglionic section produces the same effect on the ganglion cells (259). Similarly, ACh and the anti-ChE drugs have a decurarizing action at both sites. Thus, Koppanyi and co-workers (173) found that physostigmine antagonized the ganglionic paralysis produced by nicotine or curare. The same action was noted for both ACh and neostigmine by Cannon and Rosenblueth (50) and was attributed to the accumulation of sufficient ACh

to overcome the increased threshold produced by curare. Both drugs were found to augment the effects of preganglionic stimulation when given in low doses or when endogenous ACh appeared to be present at the synapse in submaximal concentrations; when administered in high dose or just prior to a tetanizing preganglionic volley their actions were depressant. Chou and deElio (53) confirmed the decurarizing action of physostigmine on the superior cervical ganglion, but under their experimental conditions obtained only a weak effect with physostigmine methiodide and none with neostigmine. They ascribed these results to the failure of quaternary compounds to penetrate cell membranes adequately. The same explanation might be offered for the findings of Schallek and Weirsma (265) on the crayfish ganglion, where physostigmine and DFP produced a block, but ACh and neostigmine had no effect on synaptic transmission. Neostigmine has also been shown to antagonize the depression of autonomic ganglia produced in dogs by dimethyl piperidines (305) and the tetraethyl ammonium ion (251).

Marrazzi and Jarvik observed that the application of DFP to the inferior mesenteric ganglion of the dog increased the number of post-synaptic fibers responding to submaximal preganglionic stimulation, without affecting the response of the non-synapsing fibers (206). Similar studies on arthropods have yielded less clear results. In the sixth abdominal ganglion of the cockroach, Roeder and coworkers (256) found that DFP produced marked facilitation and after-discharge, alternating with periods of blocking, and caused sensitization to ACh. However, neostigmine, physostigmine and strychnine produced only blocking. They suggested that the anti-ChE compounds other than DFP might have a nicotinic paralytic effect on the ganglionic cells which prevented the development of facilitation, but concluded that in this species synaptic transmission is dependent upon ChE. On the other hand, Bullock (42), investigating the properties of single synapses in the ganglion of the squid, found that DFP blocked transmission reversibly but only when applied in extremely high concentrations similar to those required for blocking axonal conduction. No hyperexcitable phase was noted. Studies on the crayfish ganglion have been noted above (265). It should be mentioned that the effects of various drugs differ markedly in these species at other sites, including the motor endplate (179-A). Koppanyi and coworkers (174, 278) compared the effects of some alkyl phosphates and physostigmine on the pressor action of ACh in atropinized dogs, as a measure of their relative effects on sympathetic ganglia. TEPP was the most active in this respect followed by HETP, eserine and DFP. The curve relating dosage to pressor effect for each of these drugs, as well as for ACh and nicotine, showed a double peak. The significance of the second phase of potentiation, at doses well above those producing inhibition, could not be explained from the data at hand.

While practically all investigators believe that ACh plays a rôle in the metabolism of the autonomic ganglion and is in some way related to synaptic transmission, beyond this point agreement ceases. Most of the pharmacological observations of the effects of anti-ChE drugs at this site can be interpreted on the basis of their acting only as enzyme inhibitors and with the full acceptance of ACh as the chemical mediator of transmission. Exceptions to this statement can gen-

erally be explained on the basis of relative penetration or some similar phenomenon. At the same time, other drugs with little or no anti-ChE activity, such as nicotine and pilocarpine, produce many of the same ganglionic effects, so that the possibility of a direct action on the ganglion cells cannot be exlcuded. It must be admitted that while the majority of the evidence at hand favors the theory, it remains to be proven. Bronk, Larrabee and coworkers (30, 185, 231), who have published numerous basic studies on the events concerned with ganglionic transmission, have pointed out that most of their findings can be interpreted by either the chemical or the electrical theory. The secondary rôle assigned to ACh in Eccles' dualistic theory of ganglionic transmission fits midway in his over-all hypothesis, according to which transmission at the neuromuscular junction is purely cholinergic and at central synapses purely electrical (84). Nachmansohn's theory (232) that transmission across the synapse is accomplished by the action current of the preganglionic fiber, with the subsequent liberation of ACh at a post-synaptic site, permits the same interpretation of the mechanism of drug action on ganglionic synaptic transmission as those postulating the release of ACh at the terminations of the preganglionic fibers.

STRIATED MUSCLE. Shortly after Feldberg and Gaddum's (99) proposal of the neurohumoral hypothesis of ganglionic transmission, evidence of a similar nature was obtained by Dale, Feldberg and Vogt (67) for the transmission of impulses from motor nerves to striated muscle fibers. Although the subsequent developments of the chemical theory for impulse mediation at these two sites have followed somewhat parallel courses, neuromuscular transmission has received more extensive study than ganglionic. This has been due in part to the fact that the neuromuscular junction is more readily accessible to experimental investigation, either animal or clinical, and that it is possible to record directly both mechanical and electrical events following the transmission of impulses to muscle fibers. Furthermore, interest in neuromuscular transmission has been stimulated by the existence of a clinical entity, myasthenia gravis, where this process is defective and the defect can be more satisfactorily explained on a chemical than on a purely electrical basis. At the present time, the ACh theory is widely accepted for transmission at the neuromuscular junction, although there are several opponents to its application to ganglionic transmission.

Following the aforementioned experiments of Dale and coworkers (67), in which an ACh-like substance was recovered from perfused striated muscle after indirect or direct stimulation, Brown, Dale and Feldberg (35), by the close intra-arterial injection of 2.0 μgm. of ACh, produced contraction of the cat gastrocnemius equal in tension to that which followed maximal indirect stimulation. Chronically denervated muscle showed a response to as little as 0.001 μgm. Small doses of physostigmine potentiated the response to ACh and converted the single twitch which normally followed a single maximal nerve volley to a brief tetanus. Curare or large doses of physostigmine or ACh produced inhibition of the response to ACh or nerve stimulation (229). Other ChE inhibitors, including methylurethanes of aromatic ammonium iodides and hordenine, were found to act qualitatively identically with physostigmine on mammalian muscle. Their potencies in this respect corresponded closely with their anti-ChE activities (12). Brown (32) recorded action potentials from whole muscles and single fibers, and found that the response to injected ACh was

a short asynchronous tetanus, the frequencies of the components of which fell off along a characteristic curve. A qualitatively similar curve was obtained for the components of the tetanus following a single nerve volley to an eserinized muscle, in which the initial intervals between spikes corresponded closely to the absolute refractory period. The effects of physostigmine were attributed to the maintenance in the muscle of a subliminal concentration of ACh. These studies were extended to denervated mammalian and amphibian muscle by Brown (33), and to avian muscle (37) and extrinsic ocular muscle (38) by Brown and Harvey. In the muscles of all these groups, the administration of physostigmine resulted in a response of contracture to ACh or to repetitive nerve stimulation, during which the initiation and propagation of excitation along the muscle fiber was blocked. This phenomenon was not observed in any other normally innervated mammalian muscle, but was considered analogous to the block of propagation obtained elsewhere with depressant concentrations of ACh, and presumably resulted from local depolarization of the fibers by the ester (38). Another effect of physostigmine noted in extrinsic ocular muscle was the apparent lowering of the threshold for direct electrical excitation during full curarization.

Harvey, Lilienthal and Talbot (141) have investigated the effects of rapid intra-arterial injection of ACh and neostigmine into the brachial artery of human subjects. Besides its vasomotor and sudorific effects, ACh produced severe pain, sensations of flexion and brief motor paresis. The injection of neostigmine resulted in more prolonged periods of localized motor paresis accompanied by visible muscular fasciculation. When similar injections were given to patients with myasthenia gravis, ACh produced a powerful localized muscular contraction, while neostigmine provoked an increase in motor power without the fasciculations or weakness noted in normal subjects (139). These and other observations (142) led the authors to postulate that the basic defect in myasthenia gravis is a reduction in the amount of transmitting agent released by nerve impulses, in association with an increased sensitivity of the muscle endplate, possibly resulting from a circulating toxic substance.

More recently, Harvey, Grob and coworkers (127, 140) have conducted similar experiments on the effects of the alkyl phosphates on neuromuscular conduction in normal human subjects and patients with myasthenia gravis. The compounds were injected into the brachial artery and the drug was kept localized by a brief period of venous occlusion. In this manner they were able to obtain maximal effects in the muscles of the forearm with a minimum of systemic side-actions. Following the administration of DFP to normal individuals there was no change in the voltage of the muscle potential in response to a single maximal motor nerve stimulus. However, the response became repetitive in nature and the initial spike was followed by a series of smaller potentials which showed progressive decline in voltage. When two maximal nerve stimuli were delivered, the voltage of the second muscle action potential was reduced. When the nerve was stimulated repetitively, the second response was greatly depressed but the third and fourth showed a remarkable recovery. This last observation is in distinction to the effects of neostigmine, where the muscle action potentials following repetitive nerve stimulation showed a progressive decline. The authors could offer no explanation for this discrepancy. The maximal effects of DFP on muscle action potentials developed within 30 to 60 minutes and were apparent for weeks. In contrast, those of neostigmine given in a similar manner developed within five minutes and were gone within an hour.

The electromyographic effects of TEPP (127) following intra-arterial injection in normal and myasthenic subjects were described as being intermediate between those of neostigmine and DFP in respect to duration of action and degree of localization. In normal subjects, TEPP produced a progressive decline in the height of the muscle action potential evoked by a train of motor nerve stimuli, similar to the picture seen after neostigmine. In other respects, the actions of the three compounds were qualitatively identical.

The intra-arterial injection of curare antagonized competely the effects of DFP on the electromyogram. Thus when the effects of the two drugs neutralized each other the muscle action potentials were of normal voltage and there were no repetitive responses (140).

The contrasting effects of DFP on muscle function in the normal and myasthenic individual should be emphasized. The normal subjects in the study of Harvey and coworkers developed fasciculation and motor weakness following the intra-arterial injection of DFP, the degree and duration of which was proportional to the dose employed. Following large amounts (2.0 mgm), pronounced paresis occurred and strength returned slowly over a period of 11 weeks. The effects of DFP on neuromuscular function in the patient with myasthenia gravis were entirely different. Before the administration of an anti-ChE the electromyogram of a myasthenic individual resembles that produced by curare. A typical depression of the second of two action potentials occurs in response to a pair of maximal motor nerve stimuli. When a train of stimuli are applied to the nerve, there is a progressive decrease in the size of the muscle action potentials. The administration of DFP corrected the abnormal electromyogram of a patient with myasthenia gravis so that the response to paired or a train of stimuli was the same as that of a normal individual without DFP. The effects of the anti-ChE were apparent within 15 minutes after intra-arterial injection and were maintained for days. No muscular fasciculation occurred and muscle strength was greatly increased (140).

The sensitization of the motor endplate to ACh in myasthenia noted by Harvey's group (194) has been denied by Acheson and associates (1) who gave intra-arterial injections of ACh in much smaller doses to myasthenic and normal subjects. Although the threshold doses for producing contraction varied greatly, they found no significant difference between the two groups with respect to either the range of threshold doses or the type of contraction. Harvey and coworkers (194) have confirmed this more recent observation. Inasmuch as fasciculation is dependent upon the synchronous firing of entire motor units (70) it was suggested that its appearance in the above studies might have been due to stimulation of some portion of the motor nerve fiber by ACh. This is in keeping with the explanation offered by Masland and Wigton, who found that the fasciculations resulting from the intra-arterial injection of large doses of ACh into the leg muscles of cats were accompanied by centripetally conducted spike potentials in the corresponding motor nerves (207). On the other hand, Eccles and coworkers (86) have demonstrated that retrograde transmission from endplate to motor nerve can occur in eserinized preparations immediately after conditioning volleys have passed through the nerve in the normal direction. Axon reflexes in-

volving entire motor units, initiated by such retrograde impulses, could also explain the fasciculation observed with massive intra-arterial doses of ACh. As would be expected, chronically denervated muscle does not exhibit fasciculation following the administration of physostigmine (182). The increase in fibrillation produced by anti-ChE drugs in such preparations (260, 223) can hardly be attributed to any specific mechanism, since the same effect results from numerous types of stimuli.

The studies of Harvey and associates on the effects of DFP and TEPP on muscle function recall to mind the syndrome of "ginger-paralysis" which was prevalent in the United States during the days of prohibition. Field investigations of the U. S. Public Health Service revealed that the paralysis occurred in individuals who drank certain lots of extract of Jamaica ginger, and M. I. Smith and coworkers (275) soon identified the offending agent as tri-orthocresyl phosphate. At that time the mechanism of action of the alkyl phosphates was entirely unknown. Now, however, the investigations of Hottinger and Block (154) have established the fact that tri-orthocresyl phosphate is an irreversible anti-ChE. Toxicity studies in dogs, cats and rabbits attest the ability of DFP to cause marked muscular paralysis and Harvey and associates have shown that a single large dose of DFP in humans can cause a paresis of long duration in normal muscle. It is possible, therefore, that the syndrome of "ginger-paralysis" is a reflection of the effects of the prolonged loss of ChE at the neuro muscular junction.

DFP has been found to act qualitatively identically with physostigmine at the neuromuscular junction of the cat (34, 157). However, when administered other than intra-arterially, relatively large doses were required to produce its effects at this site, due to the great uptake of the drug by the ChE of the plasma and other tissues. This would account for the fact that it does not appear to produce as marked fasciculations and muscular twitchings as does physostigmine after systemic administration (3).

In cats chronically poisoned with DFP, Hunt and Riker (158) observed effects similar to those seen in dogs (171), consisting of ataxia, muscular weakness and fasciculations. The altered response to intra-arterial ACh in these animals resembled that obtained after chronic denervation, being characterized by increased sensitivity to ACh and a prolongation of contraction; also, tetanus was not maintained with faradic stimulation of the nerve. Coppée and Bacq (61) found no differences between the actions of physostigmine and DFP on the isolated nerve-muscle preparation of the frog other than the failure of DFP to reinforce contractions with indirect stimulation, an effect observed with difficulty in this species after physostigmine (35, 151). Finerty (105) reported that the DFP potentiation of the effects of ACh on the frog rectus abdominus was much more marked in unbuffered Ringer-Locke solution, where the pH was 3.3, than at pH 7.0. Several factors, including the effect of pH on the rate of hydrolysis of DFP, might have played a part in producing this effect.

Guyton and MacDonald (132) made the interesting observation that the intra-arterial injection of ACh or nicotine, but not of neostigmine, produced muscular contractions in guinea pigs poisoned with botulinus toxin. This fact and the

histological appearance of the motor endplates suggested that the toxin acted by interfering with the production or liberation of ACh. The spasticity produced in the rat gastrocnemius by the local injection of tetanus toxin was found to be increased by DFP but not modified by curare or neostigmine (133).

Feng *et al.* (102, 103) and Eccles and associates (85, 86) have utilized a more basic approach to the problem of neuromuscular transmission by recording endplate potentials (e.p.p.'s) of muscle fibers stimulated via the motor nerves under various conditions. The most recent findings of the latter group have been summarized in a review (84) in which, contrary to his earlier opinion (80, 82), Eccles concludes that the transmission process at this site is purly cholinergic. Eccles, Katz and Kuffler (86) had found that physostigmine produced an increase and lengthening of the e.p.p. in curarized muscle, and with repetitive stimulation brought out a slow wave of potential at the endplate which was scarcely detectable in the absence of the drug. The endplate potential was shown to be responsible for initiating the muscle impulse (178). Although these findings were originally interpreted as being in full accord with the neurohumoral theory, further studies by Fillenz and Hanafin (104) emphasized that the slow wave was much more markedly affected by physostigmine than was the initial brief phase. This led to the adoption of a dual theory in which the slow phase was attributed to ACh, the brief phase to the setting up of catelectronic polarization by the direct effect of the action current of the presynaptic axon. A similar conclusion was arrived at by Coppée (60) as a result of his studies of transmission in the frog sartorius. However, Eccles and MacFarlane (87) have recently found both phases to react identically by several tests (curarine and ACh effects, temperature coefficients, potentiation and decline with repetitive stimulation), so that the brief phase, by analogy, is now considered by Eccles to result from the action of ACh.

Evidence suggesting that neostigmine has a direct action on the neuromuscular junction has been published by Riker and Wescoe (254). After completely inactivating the ChE of the cat gastrocnemius by the intra-arterial injection of DFP, they obtained typical normal contractions following intra-arterial injections of ACh or neostigmine. Harvey and coworkers (194) were unable to confirm this observation but do not consider their negative result conclusive. Mequel (226) reported that the complete inactivation of the ChE of the frog rectus abdominus did not prevent its being further sensitized to ACh by physostigmine or neostigmine, and concluded that both compounds acted directly on the muscle. Bacq (11) obtained a similar result with the toad but not with the frog rectus.

As Riker and Wescoe have pointed out, the chemical and pharmacological similarities between ACh, carbaminoylcholine and neostigmine point strongly to the likelihood that the last-named compound exerts a direct effect on skeletal muscle. However, it does not neceasarily follow that this is its primary effect following the systemic administration of small doses. That neostigmine does not act directly on the unstriated muscle of the iris sphincter has been mentioned (7).

The narrow dosage range in which TEPP is therapeutically effective and nontoxic in myasthenia gravis patients, compared with the much wider dosage range

possible with neostigmine, led Grob (127) to suggest that the latter drug may act by some other mechanism in addition to its anti-ChE effect. All his results with TEPP were explainable on the basis of ChE inhibition. Beck and Frommel (13) decided that neostigmine acts directly on skeletal muscle from their measurements of its lowering of the tetanizing frequency for human and guinea pig muscle, although they secured no direct evidence for such a mechanism.

An entirely different mode of action of neostigmine has been claimed by two groups. The muscle potassium content in myasthenia gravis patients, but not in normal subjects, was reported by Cummings (65, 66) to fall concomitantly with a rise in serum potassium following the injection of neostigmine. Thompson and Tice (296) obtained the opposite effects in dogs and rats, while their results with myasthenic individuals were inconsistent. However, both groups of investigators attributed the beneficial action of neostigmine in myasthenia to its effects on potassium balance rather than to its anti-ChE activity. It is quite possible that these effects were the result of epinephrine liberation (100), since the hormone has been reported to produce either a rise or fall in serum potassium depending upon the conditions of the experiment. No significant or consistent changes in serum potassium were noted in dogs following sufficient doses of DFP to produce severe nicotinic effects (172).

Curare antagonism. The long-established antagonism between physostigmine and curare and the similarity between the signs of curare poisoning and the weakness in myasthenia gravis led to the original trial of physostigmine (309) and later of neostigmine (310) in the treatment of this disease. Early quantitative studies of this antagonism by Briscoe (29) and Cowan (62, 63), and the more recent work of Bülbring (40, 41) with the isolated phrenic nerve-diaphragm preparation, have indicated that curare produces its effect, in broad terms, by raising the threshold for ACh, and that the decurarizing action of physostigmine and related drugs is directly associated with their anti-ChE activity. However, Huidobro and Jordan (156) obtained a very similar decurarizing efiect with nikethamide, a compound which has no anti-ChE activity. Huidobro's (155) studies of the effects of several other compounds on the curare-neostigmine antagonism led him to conclude that this phenomenon could not be explained adequately by the threshold hypothesis. The failure of Unna and Kimura (300) to obtain significant protections against *d*-tubocurarine poisoning in mice can be attributed to the previously mentioned observations (34, 157) that DFP localizes poorly at the neuromuscular junction following systemic administration. In rabbits, Chase and coworkers (52) found DFP to afford a prolonged definite increase in the "head-drop" dose of *d*-tubocurarine.

The studies of Eccles and associates, which have focused attention on the endplate potenital as the critical stage in neuromuscular transmission, have provided more direct information on the mechanism of curariform action. Progressive curarization was found to diminish gradually the e.p.p. following nerve stimulation until, at about one-third of its normal height, it failed to initiate the muscle impulse (178). The reasonable suggestion was advanced that curarine opposes the depolarizing action of ACh by combining with the same chemical receptors

at the endplate region (86). The additional presence of moderate concentrations of an anti-ChE drug would thus favor the transmitter action of ACh by preserving temporarily greater amounts to compete with curarine for the receptors. Whereas moderate concentrations of physostigmine alone were shown to increase the e.p.p., excessive amounts caused it to become decreased and further prolonged. The authors were inclined to attribute this effect to a combination between physostigmine and the receptors, similar to the postulated action to curarine. However, it is possible that this picture might have beeen produced by a persistent depolarizing action of excessive endogenous ACh, since polonged depolarization is also known to block transmission. Furthermore, when used in high concentrations, numerous unrelated anti-ChEs (177), as well as ACh itself (229), are "curariform" with respect to their effect on the mechanical response of muscle. Measurements of the demarcation potentials produced by various anti-ChEs at the endplate under appropriate conditions, similar to those made by Kuffler (179) with ACh and other drugs, should settle this point. These and related studies have been discussed in detail by Kuffler (179-A).

Erythroidine (276) and quinine (136) appear to be antagonized by neostigmine in the same manner as is curare. Procaine, in addition to possessing a curariform action, interferes with the liberation of ACh at the neuromuscular junction (137, 161).

The effects of curare, ACh and other drugs have been studied by means of micro-application techniques by Buchthal and Lindhard (39) and histologically by Carey (51). Reinvestigation of certain phases of their work in the light of more recent developments might prove fruitful.

The reviewers are in accord with the theory that synaptic transmission between motor nerve endings and the motor endplate of striated muscle is chemically mediated. Certainly, the actions of the anti-ChE drugs at the neuromuscular junction can most readily be explained on the basis of such a theory.

CENTRAL NERVOUS SYSTEM. The comprehensive review by Feldberg (98) which appeared in 1945 contained an extensive coverage of the literature on the actions of anti-ChE agents at central sites. Consequently, the present discussion will be concerned chiefly with publications that have appeared since then. Feldberg stated: "The present position of the theory of acetylcholine as central transmitter is all but settled." While admitting that certain facts were difficult to reconcile with the theory, he mentioned as supporting lines of evidence the presence of ACh and ChE in the central nervous system, the ability of nervous tissue to synthesize ACh, and the central effects of ACh and the anti-ChE agents. Most of the subsequent investigations have been along the same lines. One important new research tool has appeared, however, in the form of the alkyl phosphates. Results already obtained with these irreversible anti-ChE agents suggest that they will add considerably to the understanding of central nervous mechanisms.

Since Sjostrand (272) first reported on the electroencephalographic effects of applying ACh and related drugs to the cerebral cortex, several investigators have

employed this technic. The significance of such studies has been questioned by Eccles (83) on the basis of the high concentrations necessary to evoke effects and the osmotic factors involved. However, Miller (224) obtained excitation of the hypoglossal nucleus of the cat by applying bits of test paper soaked in 1:50,000,000 ACh to the floor of the fourth ventricle. The action was potentiated by physostigmine and abolished by atropine. Merritt and Brenner (221) noted that the cortical application of relatively low concentrations of ACh (0.05–0.5 per cent) plus neostigmine, or of more concentrated solutions of ACh alone, resulted in an EEG pattern in the cat which resembled that of grand mal. The effect was blocked by the subsequent application of diphenylhydantoin. The cortical suppressor areas have likewise been shown to be sensitive to the application of ACh and eserine by Becket and Gellhorn (14). The effect was manifested by a temporary diminution of electrical activity in the corresponding motor areas of the cortex, and by the failure of the latter to respond to direct electrical excitation, as indicated by electromyograms taken from the represented muscles. The authors also noted that the direct application of a purified preparation of ChE from dog pancreas to the cortex resulted in a diminution of electrical activity following afferent stimuli or the application of convulsant drugs. This latter finding is reminiscent of Mendel and Hawkins' (215) observation that the intravenous administration of a ChE preparation abolished the pupillary reflex in rats. Forster and associates (109, 110) have described a depression of cortical electrical activity immediately following the application of ACh, which was succeeded by the appearance of ACh discharges. The depression was accompanied by decreased cortical response to various sensory stimuli, and spread over the cortex in a linear fashion. It also appeared in distant areas in which subsequent ACh discharges were not apparent. The primary type of ACh discharges remained sharply localized, while secondary and tertiary discharges spread along what appeared to be neuronal paths. The significance of these observations remains open to speculation; relatively high concentrations of ACh were used.

A study by Bornstein (24) indicates that the liberation of ACh may be partially responsible for the syndrome immediately following concussion. When dogs and cats were subjected to experimental concussion under light anesthesia, the cerebrospinal fluid was found to contain abnormal amounts of ACh for as long as 48 hours afterwards. During this time characteristic behavior and EEG patterns were noted, both of which were abolished by atropine. The administration of equivalent concentrations of ACh by perfusion over the exposed cortex or by intracisternal injection resulted in similar changes which atropine likewise corrected.

Emmelin and Jacobsohn (94) have developed a technic for introducing drugs into the hypothalamic region of cats, in which the material remains confined to the limits of the third ventricle. ACh, physostigmine or neostigmine given in this manner in doses of 50 μgms produced respiratory and sympathetic effects (apnea, inhibition of motility and tone of gut and bladder, decreased volume and acidity of gastric juice, increased volume of salivary secretion) similar

to those obtained by electrical stimulation of definite hypothalamic regions. Removal of the celiac ganglion and adrenals prevented the response of the intestine but not that of the bladder, confirming the inference that the drugs acted on sympathetic centers. Similar effects were observed in human subjects by Henderson and Wilson (148) following the injection of ACh and physostigmine into the lateral ventricles. Direct evidence that ACh stimulates the cells of the supraoptic nucleus and thus causes secretion of the antidiuretic hormone has been obtained by Pickford (245). ACh (7 μgms) or physostigmine (8 μgms) injected into this region in dogs produced inhibition of urinary flow which was not obtained with injections into nearby areas or after hypophysectomy. Sensitization to ACh following denervation, which Brown and coworkers (35) demonstrated in striated muscle, occurs also in the central nervous system. Stavraky and associates (280) removed portions of a frontal lobe in cats and observed that intravenous injections of ACh then resulted in striking motor and sympathetic manifestations on the contralateral side. These effects were presumably initiated in neurones which previously had been connected with the ablated areas. When patients with lesions of the premotor and motor cortices were given intravenous injections of acetyl-β-methylcholine, similar effects were noted (107). Deafferentation of the limbs of cats sensitized the motor neurones of the anterior horns to both nervous and chemical stimulation. The chemical sensitization was not specific for ACh, however, and held for a variety of stimulants (281, 294).

Calma and Wright (48) have recently summarized the findings of several investigators concerning the central effects of physostigmine on reflexes. Potentiation occurred most frequently, although transient or predominant inhibition was not uncommon, depending upon the technics and preparations employed. In comparing intrathecal with intravenous administration in the cat, they found that the concentration in the spinal fluid had to be brought to approximately 200 times that required in the blood to produce certain reflex effects, indicating that physostigmine penetrates the white matter poorly. With the former route they obtained increase in the knee jerk, crossed extensor reflex, and jar reflex, along with an increase and prolongation of after-discharge. Effects on the flexor reflex were inconstant. Physostigmine also gave evidence of affecting irradiation, occlusion and facilitation. The last mentioned phenomenon appeared to be dependent on some degree of "long circuiting", contrary to the finding of Wikler (316) that in the cat, physostigmine produced enhancement of 2-neuron arc discharges but had little effect on multineuron arc discharges. In an earlier publication, Schweitzer, Stedman and Wright (270) obtained a close parallelism between the anti-ChE activities of a number of physostigmine-like compounds and their effects on spinal reflexes. However, most of the tertiary ammonium bases were excitatory, whereas the quaternary compounds were inhibitory in their central actions. The difference was attributed to the ability of the free bases to penetrate the cell membranes and inhibit intracellular ChE.

From studies of synaptic potentials in the anterior horn cells of the cat and frog, Eccles (81) has concluded that ACh plays no significant rôle in transmission

in monosynaptic pathways in the spinal cord. Soaking the forg or cat cord in physostigmine (1:10,000) did not modify the catalectronic potentials set up in the motoneurons by afferent impulses at frequencies of 210 to 400 per minute. Similar treatment with neostigmine and ACh was likewise without effect (83). The author believes that the synaptic potential, which is considered homologous with the endplate potential in striated muscle, is produced solely by the direct electrical effect of the action currents of the dorsal root fibers. However, these studies do not provide direct evidence of the degree of inactivation of ChE in the regions of the synapses. As mentioned above, penetration of the spinal cord by physostigmine is apparently limited (270); the quaternary compounds are probably even less permeative. It has been shown with DFP that the ChE in the central nervous system must be inhibited to a considerable extent before effects are apparent (170, 211, 236).

Gesell and associates (106, 115, 116) have advanced the theory, backed by considerable experimental work, that central and peripheral nervous integration is accomplished largely by the inhibiting effect of acid on ChE. The chief source of the acid is considered to be CO_2, which because of its high diffusibility is held to produce rapid intraneuronal changes in pH as a reflection of its concentration in the blood. Unanswered is whether under conditions of moderate hypercapnia the intracellular pH can fall to sufficiently low values to produce significant inhibition of the enzyme. The hyperpnea produced by CO_2, like that following DFP, physostigmine or neostigmine, was found to be antagonized by atropine (112). A supplemental relationship between ACh and CO_2 in the regulation of cerebral circulation has been suggested by Darrow and coworkers (68).

Slaughter and associates (273, 274) obtained marked potentiation of the analgesic effects of morphine and other opiates with small doses of neostigmine. This action was not found by Andrews (8), most of whose subjects were addicts, but was confirmed by Flodmark and Wramner (108). The latter group noted that physostigmine or neostigmine given alone also produced elevation of the pain threshold. Wramner (318) has found that the same potentiation exists in the Straub test for morphine in mice. It should be emphasized that the potentiation noted in these studies does not indicate a common mode of action of these two drugs. The effect is equally well explained by the assumption that morphine has a direct ACh-like action on the neurones involved, while neostigmine or physostigmine acts primarily by preserving endogenous ACh.

The alkyl phosphates as a group exhibit more prominent central actions than do the reversible anti-ChEs. This is probably due to the high lipid-solubility of the alkyl phosphates and the rapidity with which they gain access to nervous tissue and reduce the ChE activity of the brain to critical levels. Grob (127) found that the oil: water partition coefficient of DFP, which has marked central effects, was considerably higher than those of neostigmine or TEPP, the central actions of which are much less apparent. Many members of the series are potent convulsants and animals apparently die of central rather than peripheral actions. The convulsions are clonic and tonic and continue without interruption. It is of extreme interest that they can be rapidly and completely abolished

by low doses of atropine (117). In curarized cats and monkeys the administration of DFP produces changes in the EEG characterized by an increase in frequency of discharge and a decrease in voltage. Again these effects can be rapidly abolished by atropine or scopolamine (313).

DFP also has marked central effects in humans and these greatly detract from the therapeutic usefulness of the drug (130). Normal human subjects receiving full therapeutic doses of DFP over protracted periods develop the following symptoms referable to the central nervous system, listed in order of frequency: excessive dreaming, insomnia, jitteriness and restlessness, increased tension, emotional lability, subjective tremulousness, nightmares, headache, increased libido, giddiness, drowsiness, paresthesias, mental confusion, visual hallucinations and tremor. Changes in the EEG occur and consist of greater variations in potential, increased frequency (with increased beta rhythm), more irregularities in rhythm and the intermittent appearance of abnormal waves similar to those seen in patients with grand mal epilepsy. The increased electrical activity of the brain can be inhibited immediately by the administration of atropine. The prophylactic administration of atropine can delay the appearance of EEG changes for several weeks. However, symptoms of central origin can be prevented for only a few days and appear in the absence of abnormal electrical activity of the brain (128).

DFP also increases spinal cord activity in humans (130). This was particulaly evident in two patients with late central nervous system syphilis who had upper motor neurone lesions. These patients had spastic paraplegia of the lower extremities and no distrubance in the innervation of the upper extremities. In both, the administration of DFP resulted in intermittent and involuntary spontaneous movements of the thigh and calf muscles which were increased greatly by passive stretching of the muscles and by volunatry movement. Presumably the upper motor neurone lesions sensitized the anterior horn cells to stimulation by ACh; these patients are the clinical counterpart of the experimentally denervated animals of Cannon and Haimovici (49-A).

There is little doubt that the central actions of the alkyl phosphates are due to the inactiviation of ChE and not to a direct action of the chemicals on effector cells. No other enzymes appear to be inhibited and oxygen consumption of nervous tissue is not affected (91). It is also worthy of comment that the convulsions produced by anti-ChE drugs are the only type that can be blocked specifically by low doses of atropine. These findings by no means necessitate acceptance of the theory that central synaptic transmission is accomplished by means of chemical mediators. However, it would appear difficult to deny the fact that cells within the central nervous system possess receptors that can be stimulated readily by ACh and that this effect can be blocked by atropine and scopolamine. Indeed it would be of great interest to study further the central actions of atropine and scopolamine, particularly in relation to chemical mediation in central synaptic transmission.

Axonal Conduction. The theory that ACh functions not only in synaptic

transmission but also in impulse propagation along nerve axons and muscle fibers has been proposed by Nachmansohn and associates (43). According to the theory, the release of ACh plays an important rôle in the breakdown of membrane resistance leading to depolarization, and its hydrolysis permits repolarization. Inasmuch as the effects of anti-ChEs have been widely used, both in support of and attack upon the theory, the subject will be reviewed in some detail.

If the release of ACh is an important event in depolarization, and its hydrolysis is essential for repolarization, experimental interference with the sequence of chemical events should interrupt conduction. For example, the local application of ACh to a nerve trunk in high concentration should immediately depolarize the membrane. However, Lorente de Nó (199) observed that nerve impulse propagation was not affected by soaking a medullated nerve in a 0.9 per cent solution of ACh. Nachmansohn attributes this failure to the inability of a quaternary ammonium cation to penetrate myelin (263). To meet this objection, Eccles (83) has demonstrated that the application of 10^{-3} M solutions of ACh and an adequate concentration of anti-ChE fails to block impulse transmission during repetitive stimulation of nonmedullated fibers. He points out that there is incontrovertible evidence that ACh is liberated from postganglionic cholinergic nerve terminals and hence is able to penetrate an activated nerve membrane. Therefore, Nachmansohn's objections could not apply to nonmedullated fibers.

According to Nachmansohn's theory, the loss of ChE activity in nerve fibers should also interfere with the propagation of the impulse by leading rapidly to persistent depolarization. The demonstration that the highly lipid-soluble DFP inhibited ChE irreversibly offered a unique opportunity to investigate this point. Nachmansohn and associates (45) and Crescitelli and coworkers (64)simultaneously and independently conducted experiments based upon the premise that physostigmine and DFP should block axonal conduction, the former reversibly, the latter irreversibly. Both groups demonstrated that the application of high concentrations of either anti-ChE could block conduction, but the action of DFP could be readily reversed by washing provided the exposure was not of too long duration. To account for this, Nachmansohn and associates demonstrated that whereas the inhibition of ChE activity by DFP was immediate, irreversible inactivation was a delayed event, the duration of which was a function of concentration of inhibitor and temperature (240, 241). In a series of papers, they have related reversible and irreversible nerve block by DFP to the above functions (44, 45, 46, 97, 131). In other words, if nerves are exposed under conditions which would lead to an irreversible inactivation of cholinesterase, impulse propagation cannot be restored. On the other hand, the block produced by physostigmine can be reversed after long exposure of the nerve to high concentrations of this reversible ChE inhibitor.

The parallelism between the irreversible inactivation of ChE and irreversible nerve block loses much of its significance when one examines experiments designed to dissociate the two phenomena, a fact that has been emphasized by Eccles (84). In the experiments of Crescitelli and associates frogs were injected

with enormous amounts of DFP (2.0 grams per kilogram) and their sciatic nerves removed after one or two hours. No disturbance in conduction could be revealed when compared with control nerves, despite the fact that no ChE activity could be detected by the conventional Warburg technic. Similarly, Boyarsky and coworkers (25) exposed frog sciatic nerves to solutions of DFP (0.003 M) in peanut oil for thee hours. Conduction was unimpaired, yet no ChE activity could be detected by incubating the ground nerve with ACh and determining the rate of disappearance of the substrate by bioassay. Nachmansohn has objected to each of these experiments on the basis of the technics employed. He claims that the manometric procedure is not sufficiently sensitive to reveal the small residuum of ChE activity that would support nerve conduction. The objection to Boyarsky's technic was based upon the large amount of substrate employed relative to that which would be expected to be hydrolyzed. Nachmansohn and coworkers repeated both of the above types of experiments using the bioassay technic to determine the hydrolysis of small amounts of ACh (97). They employed only 100 μ gm of substrate per 100 mgm of ground nerve. This mixture was incubated for three hours. Solutions of ACh containing no protein served as controls. At the end of the incubation period, both types of solutions were treated with protein precipitants and the ACh content of the supernatant fluid determined by bioassay. The results indicated that enough ACh had disappeared from the experimental mixture to assign to the nerves seven to eight per cent or more of their original ChE activity. Ignored or uncontrolled by this technic was the possible effect of the protein precipitate in removing the small amount of ACh that disappeared during the procedure as well as the fact that many non-specific enzymes relatively insensitive to inhibition by DFP could readily have destroyed the ACh inasmuch as the ratio of crude enzyme source to substrate was 1000:1 on a weight basis.

Nachmansohn's criterion of the amount of ChE activity necessary to maintain conduction is also subject to interesting variations. For example, he maintains that the frog sciatic nerve can conduct normally when its ChE content is so low as to permit detection only by most elaborate procedures (97), whereas conduction in the lobster cord is markedly reduced or completely blocked when there is still 38 per cent and 21 per cent of esterase activity remaining, respectively (45). If one defines the amount of ChE activity compatible with axonal transmission as that amount which remains, no matter how small, when axons conduct, and defines the amount of ChE activity incompatible with axonal transmission as that amount which remains, no matter how large, when axons fail to conduct, there might possibly be some validity in the Nachmansohn hypothesis. However, the reviewers doubt very much that the frog nerves employed in the above experiments possessed any ChE activity and consider the methods employed by Nachmansohn to salvage some activity in support of his theory inadequately controlled.

The experiments of Nachmansohn and coworkers have been severely criticized on the basis that the high concentration of DFP or physostigmine (circa .01 M) required in the fluid bathing the nerve in order to block conduction is

many thousand times that necessary to inhibit ChE. Nachmansohn has attempted to explain this by postulating that the highly lipid-soluble DFP, which should readily penetrate myelin, is present in the nerve axoplasm in only minute amounts. To support this, he has measured the DFP content of the extruded axoplasm of squid giant axons that had been exposed to a solution containing 1 mgm per cc of the inhibitor and found it to be in the order of only 1 μ gm per cc (97). The DFP content was measured by determining the anti-ChE activity of the axoplasm. This method is entirely inapplicable inasmuch as Brauer has shown that in the reaction between DFP and ChE, both enzyme and inhibitor are destroyed. Moreover, DFP also reacts with globulins and is thereby inactivated. For example, DFP reacts with various tissue constituents so avidly that when it is injected into the radial artery it is completely inactivated by the tissues of the forearm provided there is a brief period of venous occlusion. Therefore, the technic employed by Nachmansohn would not be applicable unless a long period of equilibration permitted all the cellular consitutents capable of reacting with DFP to do so. In contrast to DFP, Nachmansohn found that eserine, which is not inactivated by reacting with ChE, distributed itself equally between the axoplasm and the fluid of the bath at concentrations up to 10^{-3} M. This is many times the concentration necessary to inhibit ChE and yet concentrations of this order or higher are necessary to block conduction. Nachmansohn explains this discrepancy by postulating a low dissociation constant of the tertiary amine within the axoplasm. Putting all of Nachmansohn's findings together one is faced with the conclusion that DFP, which is somewhat more active than eserine in blocking nerve conduction, attains a concentration within the axoplasm only 1/1000 that of eserine at the time of block. This finding is scarcely compatible with the fact that eserine is a more active inhibitor of ChE than is DFP.

A seemingly irrefutable objection to the Nachmansohn theory has been raised by Toman and coworkers (297). They stated that the logic of the ACh hypothesis demands that anti-ChEs produce conduction block by the depolarizing action of accumulated ACh. They then performed the simple, decisive experiment of measuring action potentials and demarcation potentials during the course of nerve block with DFP and eserine. Conduction failure occurred without depolarization, a phenomenon that is also characteristic of block by local anesthetics. Furthermore, they argued that according to the ACh hypothesis one would expect signs of excitation in nerves treated with sub-blocking doses of anti-ChEs. However, the least detectable action of DFP or eserine was to increase the threshold. Therefore, in common with the local anesthetics, the anti-ChEs appear to block conduction by interfering with those chemical events that lead to depolarization of the axon and not repolarization as the ACh hypothesis would demand. It is not unlikely that physostigmine acts in a manner exactly analogous to the tertiary amine local anesthetics. One could also postulate that DFP acts by the same mechanism in that presumptive evidence was presented above that eserine and DFP combine with the same moiety of the ChE molecule. The possibility exists, therefore, that the local anesthetics and eserine can react re-

versibly and DFP irreversibly with some substance in the nerve axon, the integrity of which is necessary for depolarization to occur. A common site of reaction of DFP and physostigmine with some substance other than ChE essential for axonal conduction would readily explain the observation of Nachmansohn (234) that the presence of physostigmine prevents the irreversible block of conduction produced by DFP.

The reviewers are of the opinion that the above evidence largely discredits the hypothesis that the release and hydrolysis of ACh are intimately associated with axonal transmission. On the other hand, there is little doubt that ACh can stimulate autonomic effector cells and initiate a propagated impulse in ganglion cells and at the motor endplate of striate muscle. One might generalize, therefore, that ACh is concerned with depolarizing phenomena which are non-propagated and that other mechanisms are involved in conduction.

TOXICITY. The signs and symptoms of poisoning from the reversible anti-ChEs and the methods of treatment are too well known to warrant review. Attention will therefore be focused upon the toxic effects of the irreversible inhibitors of ChE. Furthermore, the availability of the alkyl phosphates has permitted, for the first time, a study of the chronic effects of reduced tissue cholinesterase activity.

Acute toxicity. There is little doubt that the alkyl phosphates cause death by inactivating ChE and have no direct action on effector cells. Lethal doses invariably reduce tissue ChE (brain) to levels which are too low for normal physiological function (170, 211, 236). Furthermore the toxicity of the individual alkyl phosphates can be related to their activity in inhibiting ChE *in vitro* (163). The alkyl phosphates can cause death by three distinct mechanisms: (1) excessive stimulation of autonomic effector cells, (2) stimulation followed by paralysis of striate muscle and (3) central stimulation followed by depression. These will be called the muscarinic, nicotinic and central actions, respectively. The various alkyl phosphates differ with respect to the relative intensity of the three types of actions which they elicit. Furthermore species vary in their response. In the present state of our knowledge one can do little more than present in a general manner the toxic reactions to the group as a whole, and the most feasible methods for their management. The treatment of poisoning is of great practical importance because several of the alkyl phosphates are rather widely employed as insecticides.

The muscarinic actions of all the alkyl phosphates are so intense as to lead rapidly to death. Bronchial constriction and bronchorrhea are prominent features of the lethal syndrome in animals (60) but bradycardia, heart block and circulatory collapse undoubtedly contribute greatly. The muscarinic effects can be prevented by atropine. Therefore when this is the prominent feature of poisoning, atropine alone can protect animals from one or more lethal doses.

The alkyl phosphates also have a prominent nicotinic action. Excess stimulation of the motor endplate leads to muscle tremors and fasciculation. The high concentration of ACh rapidly results in muscular paralysis, and death occurs

when the respiratory muscles become involved. Protection against the nicotinic actions of the alkyl phosphates is afforded by agents which block conduction at the neuromuscular junction. Curare has not been adequately studied in this regard. However, the magnesium ion is very effective, and the parenteral administration of magnesium salts protects animals from the nicotinic actions of alkyl phosphates which otherwise would be fatal (214, 227).

Certain of the alkyl phosphates have such a prominent central action that death from minimal lethal doses results from central stimulation followed by depression. The provocative fact that therapeutic doses of atropine can prevent excessive central stimulation has already been discussed.

As an example of species differences, the response of the cat and the rabbit to DFP may be cited. The intravenous LD_{50} in the two species are 1.6 mgm and 0.4 mgm per kg, respectively (153). In the cat central and muscarinic actions are prominent and atropine alone protects 86 per cent of animals against an LD_{100} but none against two times the LD_{100}. The administration of magnesium salts alone provides only slight protection against an LD_{100}. However, a combination of Mg^{++} and atropine protects 40 per cent of animals against two times an LD_{100} (227). In the case of the rabbit, nicotinic and muscarinic actions are equally prominent. Therefore neither atropine nor Mg^{++} alone is an effective antagonist. However, a combination of both agents can save a high percentage of animals from lethal doses (214). The possibility that Mg^{++} also contributes to the control of the central actions cannot be ignored.

On the basis of animal studies, certain recommendations can be made for the treatment of human poisoning by the alkyl phosphates. Atropine should be given immediately in maximal dosage (2.0 mgm) by the intravenous route if possible. If muscular fasciculation is prominent, consideration should be given to the use of neuromuscular blocking agents. Theoretically they should stop fasciculation and increase muscle strength. However, although Harvey and associates were able to convert the abnormal electromyogram produced by DFP to a relatively normal one by the use of curare (see above), the muscular weakness was not affected, possibly because the dose of curare was excessive (140). Certainly curare should only be employed by those experienced in its use and with adequate provisions for maintaining respiration by mechanical means if necessary. The effectiveness of $MgSO_4$ in experimental animals suggests its use in human poisoning. The intramuscular injection of 10 cc of a 25 per cent solution per 100 pounds of body weight is probably a safe procedure, especially if a solution of a calcium salt is available to antagonize any excessive effect on the neuromuscular junction.

Chronic poisoning. The discovery of the irreversible anti-ChEs provided the opportunity to study the physiological effects of a chronic reduction in the concentration of tissue ChE. Such studies have been conducted in rats, dogs and monkeys, in which DFP was administered over periods ranging up to six months (171). Animals in which the level of ChE activity was only moderately depressed exhibited few signs and symptoms, and no changes occurred in the formed elements of the blood or in the blood chemistry. When dogs were given doses

sufficient to elicit nicotinic and muscarinic responses and such doses were repeated twice weekly for several months, functional disturbances of smooth and striate muscle occurred which persisted after the drug was discontinued. The first effect on striate muscle was the appearance of fasciculations in the tongue. These then spread to other muscles. Muscle weakness of the hind legs eventually leading to paralysis followed within a period of a few weeks. Complete paralysis developed within three months and showed no significant improvement after cessation of drug administration. Outstanding responses of autonomic effector cells were limited to the urinary bladder and the cardiac sphincter. The effect on the bladder was manifested by urinary incontinence. The animals developed constant dribbling and at autopsy the bladders of most were small and contracted. The action on the cardiac sphincter was the ultimate cause of death. So marked and sustained was the response at this site that the animals eventually were unable to retain even liquid food and regurgitated their undigested meals at varying periods after ingestion. Fluoroscopic and postmortem examinations revealed marked dilatation of the esophagus.

The nervous mechanism responsible for the production of cardiospasm has not been clearly defined. At least some of the fibers concerned are apparently cholinergic, since the cardiospasm produced by stimulation of the peripheral vagus in dogs was increased after the administration of physostigmine (189). That the vagus also carries inhibitory fibers is inferred by the fact that chronic cardiospasm can be produced by vagotomy (168). This subject has been discussed in some detail by Lehman (189).

The paralyzing effect of DFP on striate muscle has also been observed in cats that received either single large injections (175) or two to six successive injections (158). Fasciculations followed by ataxia and extreme muscular weakness were observed. A recurrent weakness of the hind limbs was evident for as long as 147 days. Studies of muscle function revealed an increased sensitivity to ACh and inability to maintain a tetanus.

Clinical Applications. Anti-ChE drugs are widely employed in therapy. However, discussion will be limited to their use in myasthenia gravis, abdominal distention and glaucoma, fields in which the irreversible inhibitors of ChE have received clinical trial. The results obtained to date indicate that further clinical investigations of this group of compounds will result in valued additions to the armamentarium of useful drugs.

Myasthenia gravis. In 1901, Oppenheim (244) drew attention to the similarity between myasthenia gravis and curare poisoning, in both of which conditions he considered the primary lesion to be a change in the excitability of the motor endplates. The confirmation of this general viewpoint by modern investigators has been discussed in the section on neuromuscular transmission. Six years earlier, Jolly (162) had mentioned physostigmine as a likely drug for treating the disease. Apparently this suggestion remained untested until nearly forty years later when Walker (309) administered physostigmine to a myasthenic patient and obtained temporary but marked improvement. The following year, she reported even

better results with neostigmine (310). When given in conjunction with atropine which controls muscarinic effects, neostigmine has proven to be the most valuable compound available for treating this condition (187, 243, 247, 267, 302). Of the numerous other remedies that have been tested in myasthenia (301), only three appear to be of definite benefit: ephedrine (88), potassium chloride (188) and guanidine (225). The results of trials of DFP in the treatment of myasthenia have been disappointing (59, 140). Although this compound produces an increase in strength of longer duration than that following neostigmine, the degree of improvement is less, while undesirable side-actions are more marked. The latter are referable chiefly to the gastro-intestinal tract and central nervous system, and are probably largely dependent on the high lipid-solubility of the drug. TEPP, however, has been found to have certain definite advantages over neostigmine in the clinical trials reported to date (47, 127). When the dosage schedule was properly adjusted, patients experienced approximately the same degree of muscular power as obtained with neostigmine, and it was maintained much more constantly and with less frequently administered doses. The side-effects were not nearly so marked as with DFP and were satisfactorily controlled with atropine in most patients. The chief disadvantage found with TEPP was the extremely narrow dosge range compatible with maintenance of satisfactory effects. It is to be expected that, as further members of this group are tested, still more satisfactory ones will be found.

The subjective and objective improvement which neostigmine produces is so specific in myasthenia gravis that its administration to a suspected case serves as a diagnostic test (304). Viets has modified his original test, which is dependent primarily upon clinical observation, to a more exact fluoroscopic one which is applicable when dysphagia is present (303). Another diagnostic test is based on the marked sensitivity exhibited by myasthenic patients towards small doses of curare (15).

In spite of the numerous investigations that have been aimed at elucidating the basic defect in myasthenia, it remains largely a subject for speculation. The studies of Lanari (181), Harvey (139) and others have indicated that there is a deficiency in the amount of ACh reaching the receptors of the muscle fiber following a nerve impulse. It has been frequently pointed out that this condition could result from (a) a deficiency in the amount of ACh liberated, (b) an excess of ChE at the neuromuscular junction, or (c) an increased threshold for ACh at the motor endplate, possibly due to some circulating curare-like toxin. Although no direct evidence exists in favor of the first two possibilities it cannot be said that they have been disproven (164, 291). Walker (311) has published observations favoring the circulating toxin theory. She noted that when a patient with bulbar signs exercised his forearms strenuously, no change occurred in the position of the eyelids as long as circulation in the arms was occluded by means of blood pressure cuffs. Shortly after pressure was released, the eyelids drooped markedly and the general weakness increased. Wilson and Stoner (317) confirmed this finding, and reported in addition that the serum of myasthenic patients contained an alcohol-soluble substance which blocked transmission in the

isolated frog nerve-muscle preparation. The chief criticism against this type of evidence is the fluctuating character of the signs of weakness in myasthenia, which makes the interpretation of observations during brief periods extremely difficult (194).

The relationship between myasthenia gravis and the thymus gland is most provocative. Since Laqueur and Weigert (184) reported in 1901 on the autopsy finding of a thymus tumor in a patient who died of myasthenia, several authors have noted the frequent association of these two conditions (20, 193, 246). In a routine series of 6000 autopsies, thymic abnormalities were rare (152). Thymectomy has been reported to have a favorable effect on the course of the disease (19, 54) and following the operation objective evidence has been obtained of improved neuromuscular transmission (143). However, such results must be judged conservatively because of the not uncommon occurrence of spontaneous remissions. Torda and Wolff (298, 299) reported that thymus extracts, as well as myasthenic serum, inhibited the synthesis of ACh, but this effect has been denied (290, 312). The subject of the thymus in myasthenia has recently been reviewed by Harvey (138).

Most investigators believe that neostigmine exerts its beneficial effects in myasthenia gravis by virtue of its inhibition of the muscle ChE. The injection of large doses of ACh produces a similar but extremely brief recovery of muscular strength (111). Other theories that have been advanced for the mechanism of action include a shift in muscle potassium (66, 296), a change in the accommodation curve of the motor nerves (292) and a direct action at the neuromuscular junction (254). Stare and Ricketts (279) found abnormally low O_2 consumptions in isolated muscle obtained from two myasthenic patients. Oxygen consumption was increased by the addition of physostigmine, neostigmine or certain other substances. The monograph of Goni (125) is recommended as an excellent review of myasthenia gravis.

Abdominal distention. Anti-ChE drugs have long been employed in the treatment of abdominal distention. The prominent actions of DFP on the gastrointestinal tract prompted Grob and coworkers (129) to assess the value of the irreversible anti-ChEs in this condition. The pharmacological actions of DFP on the human bowel have been discussed above. Clinically DFP has proved to be highly effective. The best results were obtained when DFP was used in conjunction with neostigmine and posterior pituitary extract. Under these circumstances the irreversible anti-ChE sensitized the bowel to the other stimulants for long periods. TEPP was much less effective in this respect (127).

Glaucoma. In Rodin's (255) account of the history of the use of physostigmine in ophthalmology, he mentions that this drug was first employed for the treatment of glaucoma in 1877 by Laqueur (183). Neostigmine has also been used for controlling intraocular tension in glaucoma, but in general has proven less satisfactory (57, 180). Leopold and Comroe (191) reported highly promising results from a trial of DFP in 78 glaucomatous eyes. A high percentage of eyes which failed to respond to physostigmine or pilocarpine was satisfactorily controlled with DFP, and in all cases, as would be expected, DFP required much less

frequent instillation than the other preparations. It is interesting to note, however, that miosis was maintained, on the average, for a shorter period following DFP in glaucomatous than in normal eyes. This raises the possibility that in glaucoma ChE may be regenerated more rapidly, or that the iris sphincter is less sensitive to ACh. The superiority of DFP to physostigmine appeared most striking in chronic simple glaucoma, glaucoma with aphakia, and glaucoma secondary to uveitis, although there were only six cases in the last category. Undesirable side-effects included visual blurring, eye and brow ache, spasm of accommodation and pericorneal injection. Occasionally, a transient rise in tension was recorded. Essentially similar results were obtained by McDonald (212) and von Sallmann and Stone (307). Dunphy (73) reported a somewhat smaller percentage of favorable responses with DFP and, while considering it the drug of choice in glaucoma with aphakia, he advised against its use in congestive glaucoma, in cases following uveitis, or in cases with shallow anterior chambers. Marr (204) considered that only 16 per cent of his cases were controlled successfully by DFP. He administered the drug only in glaucomatous eyes which were refractory to all other miotics, and established extremely stringent criteria for satisfactory control. In one case, retinal detachment occurred, presumably as the result of the marked ciliary spasm produced by DFP. Marr has obtained essentially the same results in glaucoma with TEPP as with DFP (205), but the use of the former drug led to a high incidence of irritation and sensitization of the ocular tissues.

EPILOGUE

This review cannot be concluded without a few words of apology from the reviewers. It is obvious that the actions of the anti-ChE drugs touch upon so many problems in pharmacology and physiology that a complete discussion of this group of compounds would be beyond the scope of the present undertaking. One might then pertinently inquire what dictated the choice of material. Unfortunately, no ready answer is available. In general, an attempt has been made to point out the many sites of action of ACh and anti-ChE drugs and the difficulties which arise when one tries to relate specifically a pharmacological response to the inhibition of an enzyme. Certainly a complete understanding of the reactions between enzyme and inhibitor *in vitro* and *in vivo* as well as a knowledge of the reactions of the inhibitor with substances other than the specific enzyme which it inhibits is essential before proper interpretations can be drawn. Many of the controversal issues discussed in this review depend upon such ancillary knowledge for their ultimate solution.

Those who have worked in the field of the toxicology of the anti-ChE drugs have been impressed with their high toxicity. Undoubtedly this is the result both of the great susceptibility of ChE to inhibition and the important physiological functions of the enzyme. It is obvious from the most casual studies of the potent lipid-soluble anti-ChEs that ACh has many sites of action. These include, in addition to autonomic effector cells, the motor endplate of skeletal muscle and the autonomic ganglion cells, as well as numerous other possible sites

in cortical, sub-cortical and spinal areas. The exact relationship between chemical and electrical events at many of these sites awaits elucidation. Certainly the anti-ChE drugs are destined for a major role in such fundamental studies and can provide much useful information if employed with a complete understanding of their chemistry and pharmacology.

The discovery of the anti-ChE activity of the alkyl phosphates represents a major advance in neuropharmacology and neurophysiology. Not only is their property of irreversible inhibition a unique one, but also their chemical constitution and physical properties are so different from those of the reversible inhibitors that many new pharmacological and therapeutic applications are possible. As this new group of compounds expands and is further explored it is hoped that a degree of specificity of action will be attained which will increase the therapeutic value of cholinergic drugs. Promising advances in this direction have already been made.

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