ABSTRACT

Ph.D. BIOCHEMISTRY

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HYDROLYSIS OF CARNITINE AND CHOLINE ESTERS BY CHOLINESTERASES

The hydrolysis of acetyl-dl-carnitylcholine, l-carnitylcholine and acetyl-dl-carnitine by the cholinester hydrolases was investigated. The ester bonds of acetyl-dl-carnitylcholine were hydrolyzed by the acetylcholinesterases (rat brain, human and bovine erythrocyte enzymes) to the extent of 25 per cent. The hydrolysis appeared to be stereospecific. Acetyl-dl-carnitylcholine was hydrolyzed slightly by purified human serum but not by fresh human plasma or purified horse serum cholinesterases.

Several preparations of both acetylcholinesterase and cholinesterase failed to hydrolyze l-carnitylcholine and acetyl-dl-carnitine. These three compounds, as well as several other choline esters, competitively inhibited acetylcholinesterase hydrolysis of acetylthiocholine. Acetyl-dl-carnitylcholine had marked affinity for acetylcholinesterase whereas acetyl-dl-carnitine and choline were very weak inhibitors.

The products of hydrolysis of acetyl-dl-carnitylcholine by acetylcholinesterase were acetylcarnitine and choline. The significance of these end-products in metabolism is discussed.

Hydrolysis of Carnitine and Choline Esters

HYDROLYSIS OF CARNITINE AND CHOLINE ESTERS
BY CHOLINESTERASES

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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To my father

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ABBREVIATIONS

AC	acetylcarnitine
Ac-ß-MeCh	acetyl-ß-methylcholine
ACh	acetylcholine
AChE	acetylcholinesterase
AcTCh	acetylthiocholine
ATP	adenosine triphosphate
BuCh	butyrylcholine
BuChE	butyrylcholinesterase
CCh	carnitylcholine
ChA	choline acetylase
ChE	cholinesterase
CNS	central nervous system
CoA	coenzyme A
GABA	gamma aminobutyric acid
GBB	gamma butyrobetaine
M	molar
PCh	propionylcholine
SDCh	succinyldicholine
SMCh	succinylmonocholine
TCA	trichloroacetic acid
TLC	thin-layer chromatography
Tris	Tris (hydroxymethyl)-aminomethane
µMoles	micromoles

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"The pharmacological analysis of extracts of the central nervous system is especially interesting because it seems likely that they contain unknown substances which might act as chemical transmitters" (1)

INTRODUCTION

A. Concept of Humoral Transmission

It is almost universally accepted that neurohumoral transmitter substances have a role in transmitting nervous impulses from nerves to their effector organs (2). As early as 1877, Du Bois Reymond (3) suggested that muscle contractions were excited by liberation of some chemical substance from nerves.

Elliott (4,5) reported in 1904 that the effects of stimulation of the sympathetic nervous system could be duplicated by adrenaline injection and proposed that adrenaline might be released on sympathetic stimulation.

Studies by Dixon (6,7) on vagus stimulation of the heart, led to the proposal of the release of a muscarine-like substance "inhibitin" which caused cardiac arrest. In 1914, Dale (8) tentatively identified this substance as acetylcholine (ACh), a naturally occurring chemical in ergot, which exhibited parasympathetic activity.

In 1921 Otto Loewi (9) firmly established the concept of humoral transmission. He connected two frog hearts, one of which possessed an intact vagus nerve supply. Repeated stimulation of the vagus of that heart caused release of a substance into the bathing Ringer solution which, when perfused through the second heart, inhibited cardiac contraction. The released inhibitory substance was designated "Vagusstoff" by Loewi who proposed, on the basis of its chemical and physiological properties, that it was ACh or another similar choline ester.

Subsequently Loewi and Navratil (10) found an enzyme in heart muscle extracts which inactivated "Vagusstoff" and ACh, confirming Dale's (8) discovery of the rapid hydrolysis of ACh by a blood esterase. These authors demonstrated inhibition of the action of the enzyme by the alkaloid physostigmine (eserine) (10).

Abderhalden and Paffrath (11) found an enzyme with similar actions in small intestine of horse and pig. Plattner (12) studied the ACh-hydrolyzing effects of blood but Engelhart and Loewi (13) first demonstrated the enzymatic nature of ACh destruction in blood and confirmed that minute amounts of physostigmine completely protected ACh from hydrolysis. In 1930 Plattner and Hintner (14) were first to show that such an esterase was present in nearly all animal tissues. In 1932 Stedman _et al._ (15) succeeded in isolating an enzyme, which they designated "cholinesterase", from horse serum. This impure preparation was considered a specific esterase for ACh and other esters of choline (15).

Further studies showed that this serum esterase hydrolyzed butyrylcholine (BuCh) and propionylcholine (PCh) at a rate faster than ACh (16). In fact, the cholinesterases of various sera (17) were found to hydrolyze non-choline esters of the short-chain fatty acids, although at a lower rate than choline esters (18,19,20).

1. Nomenclature of Choline-Esterases

Between 1940 and 1943 it was established that more than one kind of choline-esterase existed in animal tissues. Alles and Hawes (21,22) first demonstrated that the esterase of human erythrocytes differed substantially in substrate specificity and other characteristics from the serum esterase. They showed that the erythrocyte enzyme which hydrolyzed ACh faster than other choline esters, was inhibited by excess ACh; and it hydrolyzed both acetyl-α-methylcholine and acetyl-ß-methylcholine, the latter being unaffected by the serum enzyme.

Mendel and others (23,24,25) showed that these two enzyme types differed in their distribution, their sensitivity to inhibitory agents, and their optimum substrate concentrations. In addition, the appearance of the pharmacological effects of their inhibitors was related to inhibition of the erythrocyte-type enzyme.

Investigation of the substrate specificities of the cholinesterases (ChE's) of animal tissues led Mendel and Rudney (23,24,25) to propose a general classification of the enzymes using specific substrates - acetyl-ß-methylcholine for the "erythrocyte" esterase and benzoylcholine (BzCh) for the "serum" enzyme. They named these two enzymes "true" ChE and "pseudo" ChE, respectively.

In 1942 Richter and Croft (26) showed that ChE's could be differentiated from simple esterases by their marked sensitivity to 10^{-5}M eserine.

Further research demonstrated that the "true" ChE included enzymes from erythrocytes and nervous tissues which hydrolyzed ACh, PCh, and acetyl-ß-methylcholine. Since "true" ChE did not hydrolyze BuCh or BzCh, Nachmansohn (27,28) suggested the name acetyl-cholinesterase (AChE) for these enzymes. "Pseudo" ChE, on the other hand, which is found in serum, pancreas, and other tissues hydrolyzed ACh, BzCh, and BuCh. The name butyrylcholinesterase (BuChE) was suggested for this esterase (29).

For the discussion which follows, the nomenclature of the I.U.B. Commission of Enzymes (30) will be used. Acetylcholinesterase (AChE) (28) is the name of acetylcholine acetylhydrolase (EC 3.1.1.7.), formerly known as true cholinesterase (ChE) (25), specific ChE (31), e-type ChE (32), Type I ChE (33,34), or aceto-ChE (35). Cholinesterase (ChE) is the trivial name of acylcholine acylhydrolase (EC 3.1.1.8.), formerly known as pseudo-cholinesterase (25), unspecific ChE (31), non-specific ChE (31), non-specific acetyl-ChE (28), s-type ChE (32), Type II ChE (33), or x-ChE (34).

Cholinester hydrolase is used to designate choline ester splitting hydrolases in general, since "cholinesterase", formerly used in that sense, is inconsistent with the newly accepted nomenclature.

When necessary, distinction is made between butyrylcholinesterases and propionylcholinesterases according to the substrate which is hydrolyzed most rapidly.

The simple classification of cholinester hydrolases into two types has been subject to criticism. For example, in 1946 Bodansky demonstrated that human erythrocyte AChE (36) and mouse brain AChE (37) hydrolyzed triacetin at a rate faster than ACh. The ChE of horse serum hydrolyzed methyl butyrate and tributyrin, while AChE did not (38). Swine serum ChE did not hydrolyze BzCh (39) whereas chicken serum ChE hydrolyzed both acetyl-ß-methylcholine and BzCh (40). Certain tissues were found to have a benzoyl ChE distinct from ChE (41). Guinea-pig liver contained a benzoyl ChE (BzChE) distinct from serum ChE (42) which was resistant to 2×10^{-6} M eserine, as was the serum of rabbits (43). Ord and Thompson (44,45) demonstrated in rat heart and brain tissues the presence of a ChE which exhibited maximum activity with propionate esters. For these reasons, the more specific terminology is favoured.

2. Identification of Acetylcholine in Tissues

Most of the methods which have been used for the identification of ACh in tissue extracts have lacked specificity. Dale and Dudley (46) first isolated ACh from ox spleen and Stedman and Stedman (47) identified ACh in ox brain in 1937, although they discarded 99% of the active material originally isolated. This identification was questioned when Banister *et al.* (48) found that the ACh-like activity of ox spleen was, in fact, composed of three components - ACh, PCh, and the 'F' component, later suggested to be a mixture of ACh, PCh, and a zwitterion (49). Beznak (50) reported that rat hearts contained material which appeared to correspond to this 'F' component, and which was tentatively identified as an ACh complex.

Other choline esters have also been found in brain (51) and other tissues (55,56,57), and the possibility of other substances having ACh-like activity in tissue extracts has been widely suggested (48,52,53,54).

The amounts of ACh-like material obtained in various animal tissues vary according to the method of extraction but recently Toru and Aprison reported a very efficient extraction procedure for brain "ACh" which yielded a concentration of 31.2 ± 1.5 mμmoles ACh/g adult rat brain (excluding the cerebellum, pons and medulla oblongata) (77).

The identification of "ACh" has been called into doubt by several investigators (48,50,52,53,54,58). "ACh" is widely occurring and its presence in many tissues, such as placenta and spleen, remains unexplained. The usual method of identifying ACh has been that of parallel quantitative bioassays (46) although even Chang and Gaddum pointed out that such assays, on as many as five different test preparations, failed to distinguish pyruvylcholine from ACh. Thus the presence of pyruvylcholine in a mixture with ACh would escape detection (58). More recently, Hosein and Koh have found that the method of parallel bioassay cannot be used to identify ACh in mixtures of substances with ACh-like activity such as tissue extracts (59,60).

Results from partition coefficient and colorimetric experiments led Hosein et al. to propose that "bound" ACh was, in fact, not entirely, nor even primarily, ACh (61) and Henschler, in studies of royal bee jelly, questioned the identification of ACh by paper chromatography (62).

Using a choline-acetylating system from rabbit brain in 1949, Nachmansohn et al. synthesized a compound with ACh-like activity but chemically distinct from ACh (63,64). Brain materials with ACh-like activity which were not affected by the cholinester hydrolases have been reported (54,65,66), and various ACh-like compounds have been isolated from invertebrates (67). Recently bovine brain extracts were fractionated into 89 subfractions, 6 of which exhibited ACh-like activity (68).

Giarman and Pepeu (69) found that 90% of their estimated "ACh" was due to ACh or "a very closely related derivative" and Curtis and Andersen suggested that a compound structurally related to ACh but not ACh itself was the transmitter of the ventrobasal complex of the thalamus (70). Szerb also stated that "ACh or a very similar ester is the transmitter at the cholinergic synapses in the cortex of the brain" (71). Several attempts have been made recently to identify chemically the ACh-like substances of tissues (72,73,66), including the gas chromatographic method of Stavinoha et al. which distinguishes acetic acid esters from other esters (74,75). Maslova (76) showed by polarographic estimation of the ACh content of rabbit ganglia that the actual weight of ACh present in such extracts was only 20% of that which was determined by bioassay.

Thus the nature of the ACh-like material in animal tissues is still very much in question.

(i) Other Esters of Biological Origin

Other choline esters have been discovered in animal tissues (78). They included acrylylcholine (79), senecioylcholine (80,81,82), urocanylcholine (83,84), imidazolylacetylcholine (85), palmitoylcholine (86), and other unidentified esters of choline (87,88,67) as well as PCh (55,89, 48,90,54) and BuCh (51,91,92,93).

The physiological functions of these esters, in particular PCh and BuCh which are very widely distributed and pharmacologically active, are not known.

(ii) GABA-Choline

Kuriaki *et al.* (94) synthesized γ-amino-butyrylcholine (GABA-choline) and suggested its presence in brain as an inhibitory neurotransmitter. Studies on the inhibitory activity of synthetic GABA-choline (95) supported this idea and presently both GABA and GABA-choline are believed to be involved in inhibition in the CNS (96,97). Kewitz (98) identified GABA-choline in extracts of pig brain and demonstrated its hydrolysis by ChE.

(iii) Succinylcholine

The dicholine ester of succinic acid (succinyldicholine, SDCh) is a potent muscle relaxant widely used today in anaesthesia. Several groups of investigators have studied the prolonged apnoea in certain patients following the injection of normal doses of SDCh and in most cases they found abnormally low levels of serum ChE (99,100,101,102,103).

SDCh acted on the post-junctional structures of the neuromuscular junction and was also found to affect the motor nerve terminal (104). A second explanation for delayed recovery from SDCh muscle relaxation was that it might be due to the presence of succinylmonocholine (SMCh), itself a relaxant drug (105). Anticholinesterase drugs, such as eserine, greatly prolonged the pharmacological effects of SDCh, suggesting that the drug is destroyed by ChE (106,107).

Davies and Kalow (108) and Whittaker and Wijesundera (109) carried out extensive studies of the separation of SDCh and its hydrolysis products by paper chromatography. Whittaker and Wijesundera (57, 110) used this method to follow the *in vitro* hydrolysis of SDCh by horse serum ChE, by separation of the constituents of the reaction mixture at different stages of enzymatic hydrolysis (110).

They demonstrated that the hydrolysis of SDCh by purified horse serum ChE occurred in two stages, SDCh being hydrolyzed first to choline and SMCh. The SMCh was then hydrolyzed at a very much slower rate to choline and succinic acid but no succinate was detectable until all the SDCh had been hydrolyzed.

SDCh was a competitive inhibitor of ACh hydrolysis by both AChE and ChE, being a much better inhibitor of the former (100).

SMCh was hydrolyzed by serum ChE but as a substrate of erythrocyte AChE it occupied a position between ACh and SDCh, i.e., it was a moderately good substrate. SDCh was a good inhibitor of AChE hydrolysis of ACh even at low concentrations, whereas SMCh inhibited only at high concentrations (100,111).

The different affinities of SDCh and SMCh for horse serum ChE were presented by van Hees et al. (112).

Substrate	ACh	SDCh	SMCh
K_m	1.8×10^{-3} M	1.8×10^{-3} M	2.9×10^{-2} M

3. Butyrobetaines and Related Esters

The discovery of the butyrobetaines in animal tissues parallelled that of their structural analogues, the choline derivatives.

Carnitine was first discovered in 1905 in muscle extracts (113, 114). Tomita and Sendju found its structural formula to be γ-trimethyl-ß-hydroxybutyrobetaine, the levorotatory isomer being the naturally occurring form (115).

Krimberg (116) pointed out the relationship of carnitine to γ-butyrobetaine (GBB), a constituent of a variety of animal tissues. GBB was implicated as the toxic compound of decaying horse meat which was responsible for the excessive cholinergic activity in dogs fed such meat (117). Friedman *et al.* (118) isolated GBB and identified it in meat extracts.

Linneweh (119,120) isolated crotonbetaine from animal tissues and demonstrated the interconversion of GBB, crotonbetaine and carnitine in dog tissues. He extended Engeland and Kutscher's (121) observation of the following metabolic pathway:

Protein → glutamic acid → γ-aminobutyric acid → GBB

and postulated a further pathway which recalled Knoop's theory of fatty acid oxidation.

GBB → crotonbetaine → carnitine → breakdown products.

Hosein, Proulx, and Ara (54) subsequently suggested that these butyrobetaines were naturally occurring substances in the form of their coenzyme A esters.

Pharmacological studies have been carried out on these quaternary ammonium compounds and their synthetic esters to determine whether or not they possess cholinergic activity. Kutscher and Lohmann (122) found weak cholinergic activity for carnitine on several preparations whereas Linneweh (120) found carnitine to be ineffective. He found, on the other hand, that GBB and crotonbetaine possessed weak curare-like properties (120). Hosein and McLennan studied the pharmacological actions of GBB and found parasympathetic excitation (123) which was greatly enhanced in the synthetic methyl and ethyl esters of GBB (123). The esters were not attacked by the cholinester hydrolases (124,129). Hunt and Renshaw found that GBB was inert (125,126) and Hosein and Proulx (127) reported the isolation of GBB from brain extracts of convulsed animals, but not from those of normal animals. Strack and Fosterling (128,129) demonstrated that, while crotonbetaine was cholinergically inactive, its methyl and ethyl esters were very active.

Charlier (130) reported the cholinergic activity of the synthetic compound carnitine carnitate, or bicarnitine.

Hosein (131) reported the isolation of GBB, crotonbetaine, and carnitine from rat brains during drug-induced convulsions and later showed that the "free" ACh of brain extracts consisted of ACh and PCh, while the "bound" fraction contained, in addition to the choline esters, a mixture of the coenzyme A ester derivatives of the butyrobetaines (132, 53, 133). In 1964, the isolation of acetyl-l-carnitine from calf brain was also reported (134). It was found that the increased ACh-like activity of the brains of narcotized rats was due, in very large part, to the presence of acetyl-l-carnityl CoA, a substance not susceptible to the actions of the cholinester hydrolases (135, 136, 139).

Acetyl-l-carnityl CoA is γ-trimethylammonium-β-O-acetyl-butyryl CoA. The enzymatic synthesis of the compound was carried out using acetyl-l-carnitine and CoA-SH as substrates for the fatty acid activating enzyme (FAAE) of beef heart (137). Hosein and Smoly (138) proposed that this compound might be a precursor of acetyl-l-carnitylcholine (ACCh).

(i) The Physiological Functions of Carnitine and Acetylcarnitine

Strack and others (140,128,122,129,141) have endeavoured to discover the physiological functions of carnitine and acetylcarnitine (AC) in animal tissues.

Both carnitine and AC were not cholinergically active on frog heart, frog rectus, leech dorsal muscle and mouse ileum preparations (142) although Fritz (143, 144) demonstrated cholinergic properties of dl-AC when it was injected directly into rat brains.

Carnitine enhanced the transport of acetyl- and acyl-CoA moieties across mitochondrial membranes (143, 145) thus regulating the rate of fatty acid oxidation (146) and fatty acid synthesis (147, 148). Carnitine was not found to be involved in transmethylation reactions (149) nor could it substitute for choline (150, 151). Thus much evidence supported the role of carnitine as a shuttle for the transport of long chain acyl groups to the intramitochondrial fatty acid oxidation site from extramitochondrial sites (152, 153, 146, 143).

The reversible enzymatic acetylation of carnitine was studied in 1955 by Friedman and Fraenkel (118), and evidence has been produced that in kidney mitochondria there was a specific AC stimulation of oxidation of endogenous fatty acids (154). This is of particular interest as the O-acyl structure of acylcarnitine derivatives has a high energy group potential (155), unlike ACh.

Although Pearson and Tubbs (156) suggested that AC "may be a metabolic backwater", in certain microorganisms carnitine is a vitamin (B_t) while in others it is dehydrogenated to 3-dehydrocarnitine and then further metabolized to glycine betaine (157). Of interest, moreover, was the discovery that rat tissues, in particular heart muscle in which the concentration of AC was very high (156), decarboxylated l-carnitine to ß-methylcholine. There may be a physiological requirement for ß-methylcholine or acetyl-ß-methylcholine which is known to have specific cholinergic receptors (159) in the heart (158,160,161,162) and which is rapidly hydrolyzed by AChE (25).

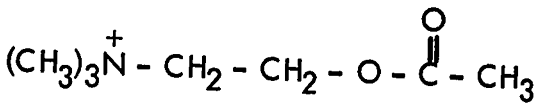
(ii) Structural Similarities Between Butyrobetaine and Choline Esters

The structural similarities between these two groups of quaternary ammonium compounds are illustrated below:

Choline Esters

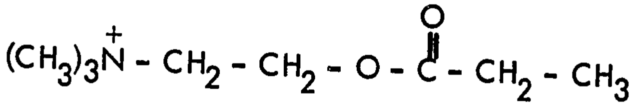
1 2 3 4 5 6

ACh



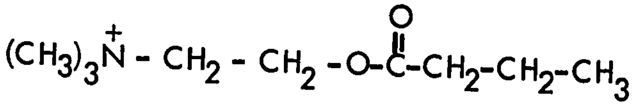
$(CH_3)_3\overset{+}{N} - CH_2 - CH_2 - O - \overset{\overset{O}{\|}}{C} - CH_3$

PCh



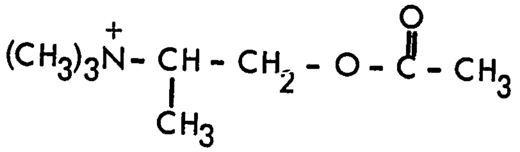
$(CH_3)_3\overset{+}{N} - CH_2 - CH_2 - O - \overset{\overset{O}{\|}}{C} - CH_2 - CH_3$

BuCh



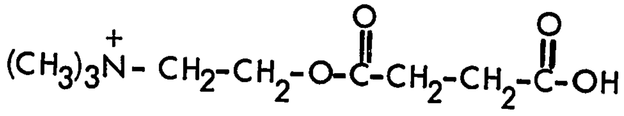
$(CH_3)_3\overset{+}{N} - CH_2 - CH_2 - O - \overset{\overset{O}{\|}}{C} - CH_2 - CH_2 - CH_3$

Ac-ß-MeCh



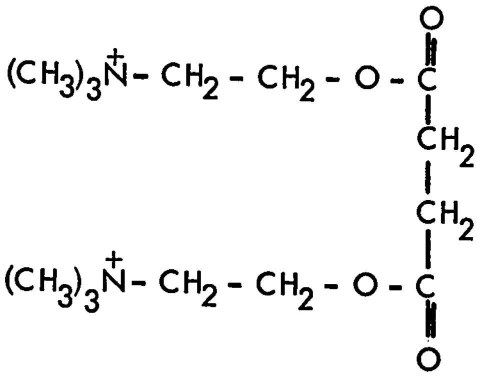
$(CH_3)_3\overset{+}{N} - \underset{\underset{CH_3}{|}}{CH} - CH_2 - O - \overset{\overset{O}{\|}}{C} - CH_3$

SMCh



$(CH_3)_3\overset{+}{N} - CH_2 - CH_2 - O - \overset{\overset{O}{\|}}{C} - CH_2 - CH_2 - \overset{\overset{O}{\|}}{C} - OH$

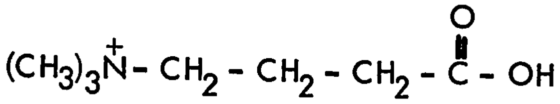
SDCh



$$(CH_3)_3\overset{+}{N}-CH_2-CH_2-O-\overset{O}{\overset{\|}{C}}-CH_2-CH_2-\underset{O}{\underset{\|}{C}}-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$$

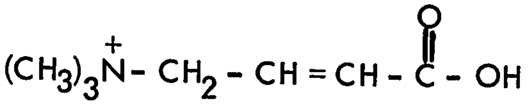
Butyrobetaines

GBB



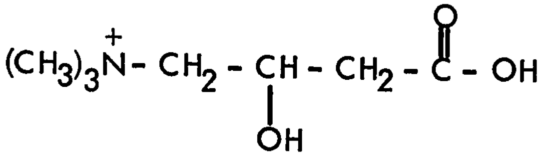
$$(CH_3)_3\overset{+}{N}-CH_2-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-OH$$

Crotonbetaine



$$(CH_3)_3\overset{+}{N}-CH_2-CH=CH-\overset{O}{\overset{\|}{C}}-OH$$

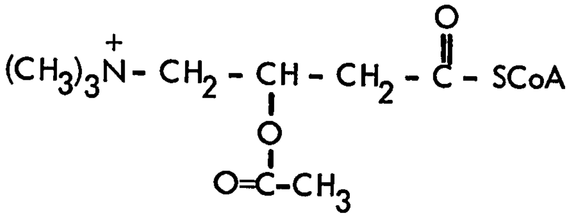
Carnitine



$$(CH_3)_3\overset{+}{N}-CH_2-\underset{OH}{\underset{|}{CH}}-CH_2-\overset{O}{\overset{\|}{C}}-OH$$

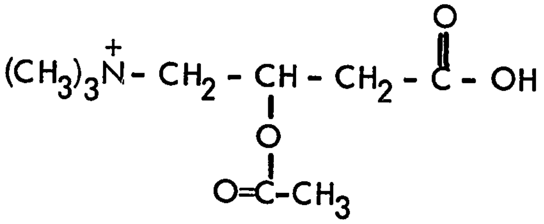
Butyrobetaine Esters

AC-CoA



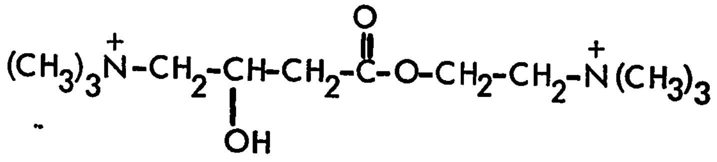
$$(CH_3)_3\overset{+}{N}-CH_2-\underset{\underset{O=C-CH_3}{|}}{\underset{O}{\underset{|}{CH}}}-CH_2-\overset{O}{\overset{\|}{C}}-SCoA$$

AC



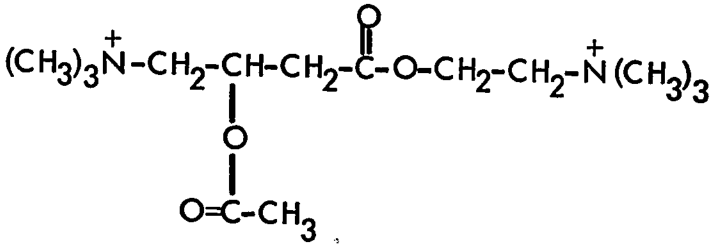
$$(CH_3)_3\overset{+}{N}-CH_2-\underset{\underset{O=C-CH_3}{|}}{\underset{O}{\underset{|}{CH}}}-CH_2-\overset{O}{\overset{\|}{C}}-OH$$

CCh



$(CH_3)_3\overset{+}{N}-CH_2-\underset{OH}{CH}-CH_2-\overset{O}{\overset{\|}{C}}-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$

ACCh



$(CH_3)_3\overset{+}{N}-CH_2-\underset{\underset{O=C-CH_3}{O}}{CH}-CH_2-\overset{O}{\overset{\|}{C}}-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$

Comparison of these quaternary ammonium compounds demonstrates their similarity of structure. The butyrobetaines differ from the choline esters in possessing a carbon atom at position 4 instead of an oxygen atom. It may be noted that the AC, AC-CoA, ACCh, SMCh, and SDCh all contain within their backbone the entire ACh molecule.

The importance of molecular structure of quaternary ammonium compounds with respect to their cholinergic activity has been discussed by Pfeiffer (163), Ing (164), Welsh (165), Beckett et al. (166) and Barlow (167). For a compound to exhibit cholinergic activity (i.e., the biological activity expressed relative to that of ACh on the same test preparation), it required both a quaternary ammonium group and a carboxyl radical (168, 169). Pfeiffer suggested that muscarinic activity resided mainly in the ester link, whereas the quaternary nitrogen effected primarily the nicotinic activity of ACh-like compounds. For these reasons AC and other butyrobetaine esters would be expected to have ACh-like activity.

Pfeiffer (169) suggested, moreover, that a neurotransmitter compound possessing in its structure a ß-carbon substituent would have greater muscarinic activity than ACh. AC, AcCoA and ACCh all possess a ß-substituent whereas ACh does not. Of interest is the discovery of an enzyme which decarboxylates l-carnitine to ß-methylcholine (158), which is related to acetyl-ß-methylcholine, a compound having specific cholinergic receptors in the heart (159).

4. Enzymatic Synthesis of Choline Esters

Comprehensive reviews of the distribution, nature and mechanism of action of choline acetyltransferase (E.C. 2.3.1.6.) have been published (174, 175, 176, 187).

In vitro synthesis of ACh was first demonstrated in mammalian brain slices by Quastel *et al.* (170). Subsequently enzymatic synthesis was shown to occur *in vitro* *in* several tissue preparations (47, 171, 172). Nachmansohn and Machado (173) determined the requirements of the ACh synthesizing system to be ATP, choline, K^+, Mg^{++}, and "active acetate", later shown to be acetyl coenzyme A. These authors named the enzyme "choline acetylase" (ChA). The acetylation proceeds in a two-step mechanism consisting of the formation of acetyl CoA, followed by the transfer of the acetyl moiety to the choline hydroxyl oxygen. For this reason, Korey *et al.* (178) proposed that the name choline acetylase be reserved to describe the enzyme which catalyzes the second acetylation reaction.

Several authors have proposed that ChA is localized inside the synaptic vesicles (189,190). Others observed that guinea pig brain ChA is freely dissolved in the cytoplasm of the nerve endings (171), since most of the ACh-like material and ChA of brain homogenates was isolated in a fraction of pinched-off nerve endings, or synaptosomes (183,184,185). Still others have suggested that structures other than synaptic vesicles are responsible for the binding of ChA in the nerve endings (180,191,192). For example, the earlier findings of Hebb and Smallman (180) which showed that 50–70% of the enzyme was attached to the particulate mitochondrial fraction of brain tissue and that 21-28% was present in the soluble supernatant fraction, might have been due to an artifact.

The source of the precursor choline has also been investigated. There was no evidence that nerve terminals recaptured the ACh they released, but Perry (186) demonstrated that nerve terminals in ganglia did take up choline from the medium. Recently Friesen *et al.* (73) showed that stored choline and phospholipid choline were insufficient in stimulated preganglionic nerve terminals to maintain adequate ACh synthesis indicating possible reuse of the choline.

(i) Specificity of Choline Acetyltransferase

In 1949 Nachmansohn *et al.* (63) reported that another choline ester was synthesized by the same enzyme system as ACh. Although the ester was pharmacologically similar to ACh, it differed from it chemically. This evidence indicated that ChA was not specific for the synthesis of ACh. In fact, ChA is note-worthy for its lack of specificity. Lack of specificity for the acyl group was demonstrated by Nachmansohn et al. (178,181) who showed formation of PCh as well as ACh by squid ganglia. Others demonstrated the formation of PCh or BuCh by pigeon brain and squid ganglion enzymes when the acetate in the incubation medium was replaced by propionate or butyrate (178,55).

Korey, de Braganza and Nachmansohn (178) showed a decrease in the rate of esterification with increase of acyl chain length from 2 to 4 carbon atoms. More recently Berry and Whittaker (182) identified three types of acylating enzymes: ChA I, synthesizing ACh and PCh; ChA II, synthesizing acylcholine esters from C_4 to C_6; and ChA III synthesizing palmitoylcholine. Non-aliphatic esters were formed from pyruvic, lactic, nicotinic, and glutamic acids (178).

The lack of specificity toward the choline molecule was also studied by Korey et al. (178) who found that squid ganglion ChA acetylated both dimethylethanolamine and choline at the same rate. Burgen et al. also studied the effects of modification of the choline structure on enzyme activity and found that of 25 substrates tested, choline and 16 other analogues were acetylated by the enzyme preparation(174).

Hosein and Smoly (138) demonstrated that when the quaternary ammonium compound, acetyl-l-carnityl CoA (from (139) narcotized brain extract) and choline were incubated in the presence of rat brain ChA, acetyl-l-carnitylcholine (ACCh) was formed.

5. General Concept of Cholinergic Transmission

The general concept of transmission by ACh has been extensively reviewed (195,196,197,198). ACh is thought to be bound in quantal units (193,194) to a storage component of the cell, which is probably a protein (174). It is held in a bound form until released by bioelectric events such as a nerve impulse (195,196). The released ester reacts with a specific receptor and possibly induces a conformational change in the post-synaptic receptor protein (196). According to Nachmansohn (196), this alteration in receptor configuration is responsible for a change in ion conductance, particularly of Na^+ and K^+, and this leads to depolarization of the postsynaptic membrane potential. AChE is responsible for the termination of the ACh activity by rapidly hydrolyzing the free ester (195,196), thus allowing the receptor to return to its resting configuration and the return to the resting postsynaptic membrane potential (195).

(i) The Cholinergic Receptor

The nature of the cholinergic receptor has recently been reviewed (198). It is generally assumed that the first step in ACh action is the formation of a reversible complex between ACh and a membrane component called the ACh "receptor" (199). Nachmansohn proposed that this structure was a protein, the combination of which with ACh caused a conformational change with a shift of charge leading either directly or through some secondary steps to ionic permeability changes (200,201). Such conformational changes following interaction of proteins and small molecules have been documented (202,203).

One might expect that any substance which reacted with the receptor would also react with AChE, in as much as many investigators have indicated a structural similarity for the two proteins (204). In fact, several workers suggested that AChE and the ACh receptor were one and the same protein and that the two active centres were either fully or partially identical (205,206,207,208,198).

This proposal was incompatible with several experimental observations. The oxygen, sulphur, and selenium isologues of ACh, although isosteric, differed in their abilities to induce changes in AChE and the ACh receptor (212). This was concluded from observations that the pharmacological activities of these compounds on the eel electroplax (209) and other preparations (210) differed markedly, whereas all these compounds were hydrolyzed at very similar rates by eel AChE (211). Karlin (213,214) demonstrated strong inhibition of the ACh receptor by compounds reacting with both sulphydryl and disulphide groups under similar conditions in which no effect on the K_m or V_{max} of AChE was noted. He concluded that these were two separate proteins.

Another approach to the problem was that of Beckett et al. who proposed a possible muscarinic receptor model by studying the conformation of several muscarinic cholinergic substances with a high degree of complementarity to ACh (215). Similarly, Pfeiffer (163) attempted to relate the spatial relationship of the prosthetic groups of parasympathomimetic drugs with a theoretical muscarinic receptor.

Studies of several series of bis-quaternary compounds indicated that a distance of approximately 14 Å between the two basic nitrogen groups was optimal for muscle relaxant activity while an interquaternary distance of 6-7 Å was optimal for ganglionic blocking activity (216). This indicated a difference in the receptors of these two tissues and suggested that the use of specific compounds was of value in mapping the receptive surface of proteins.

B. The Cholinester Hydrolases

1. Methods of Cholinester Hydrolase Determination

Reviews of the several methods of cholinester hydrolase determination have been published (217,218,219). Both AChE and ChE can be assayed (a) manometrically as CO_2 liberation from a bicarbonate buffer, (b) by measurement of a change of pH by electrometric or colorimetric means, (c) by titrimetric methods for the acids liberated using indicators or a pH meter, (d) colorimetrically determining ester concentration, or (e) colorimetrically using coloured substrates. Neither ACh nor its hydrolysis products, choline and acetic acid, can readily be estimated spectrophotometrically. Recently Stein and Lewis have suggested automated methods for the study of AChE (220).

Alternatively, in a method described by Burn (221), the destruction of ACh can be followed by bioassay on the frog rectus preparation. This method is very sensitive (permitting determination of substrate concentrations of 5×10^{-7}M) but it is very tedious. For routine experimental work it is easier to follow ACh hydrolysis chemically.

2. Subcellular Localization of the Cholinester Hydrolases

According to Nachmansohn, AChE is present in the conducting tissues of the animal kingdom, in all types of nerves. The enzyme is localized at the neuronal surface where its biological functions are thought to take place (222).

There are indications that AChE is synthesized in the perikarya of neurones (223,236) and that it spreads from there into the neuronal processes (237). The distribution of the internal cytoplasmic AChE has been shown to resemble that of Nissl substance. The presence of AChE throughout the entire length of cholinergic neurones (238,239) may be due to its being in transit to its functional site. Weiss and Hiscoe (224) proposed a process of proximo-distal movement of axonal proteins to the periphery (225), although more recent work has indicated that AChE may be synthesized locally in the axon of peripheral nerve (226,227).

Most of the AChE activity in brain tissue homogenates is associated with the subcellular particles. The AChE activity of rat brain was distributed primarily between the mitochondrial and microsomal fractions (228, 229,230), whereas the ChE was distributed largely between the nuclear and soluble fractions (228,229,230). Results from studies with the electron microscope indicated that AChE was associated with membranous structures (228,231), particularly those of the synapses and nerve endings (232,233).

Giacobini (234) has estimated the AChE activity of single neurones using a microgasometric diver technique. In the anterior horn cell of the rat spinal cord he found that the cell body exhibited the highest AChE activity, the axon and dendrite somewhat lower values and the nucleus had very little activity (235).

Because of the great variety of cell species within the brain, it has been suggested that homogenization and differential centrifugation studies may be of questionable value in the investigation of brain components (228,235).

3. Distribution of the Cholinester Hydrolases

(i) Nervous System

Cholinergic nerve fibres have been identified by their distribution of enzymes controlling ACh metabolism (266), particularly AChE. Generally the correlation between the amounts of the esterases, ACh and ACh synthesis was good, the exceptions being the cerebellar hemispheres with very high AChE and the anterior spinal roots with very low AChE (244). No theory of AChE function has explained these anomalies, nor the presence of AChE and ChE in many non-nervous tissues.

Very early studies showed that the brain contained AChE (25,27,243) and that grey matter hydrolyzed ACh most actively (240). It also contained ChE (33,243,244,91), especially in the white matter. The relative amounts of AChE and ChE of whole rat brain homogenates were found to be 94% and 6%, respectively (241). The ChE of vertebrate brain was largely located in the capillary endothelium and in the neuroglia (263) but among the different cell types of the CNS there was a continuous range of enzyme concentrations of both AChE and ChE (264). For example, Hebb reported a very marked species variation in the distribution of the two enzymes in the cerebellum. ChE was discovered in some Purkinje cell bodies, indicating that the old theory that only AChE is found in neurones and only ChE in glia and blood vessels (268,92) was wrong. Both ChE and AChE increased in brain and spinal cord of rats after birth (249,250) until functional and morphological maturity and no neurones lacking the ability to hydrolyze ACh were found within the cat spinal cord (248).

Peripheral nerve contained both enzymes (241,242,41). The ChE was found mainly in the Schwann cells of myelinated fibres (238), such as the giant squid axon (246). ChE was evenly distributed in the neurolemmal sheathes of the guinea pig sciatic nerve (262) and in the rat dorsal roots (35).

AChE was found in the nerve fibres and synapses of the CNS (247) and was localized in the axonal membranes of frog nerve fibres (258, 259, 262). Marked AChE activity was noted at the Ranvier nodes of myelinated peripheral nerve fibres (260) and the gradient of AChE in the phrenic nerve suggested that AChE was metabolized not only at nerve terminals but also in the axon (261).

The cells of all parasympathetic ganglion neurones demonstrated high AChE activity (239) and in the parasympathetic ciliary ganglion of the cat, Koelle showed AChE both pre- and postsynaptically (239). AChE was also found in all ganglion cells of the rat sympathetic superior cervical ganglia (254,255), the intensity of activity ranging from weak to strong. The preganglionic nerve fibres stained more strongly for AChE than did the postganglionic fibres (254), and Sjoqvist (257) concluded that in such sympathetic ganglia, cells with moderate to high levels of AChE were from cholinergic neurones while the majority with weak activity were from adrenergic neurones.

ChE also occurs in the autonomic ganglia (242,35), probably in the interstitial tissue and capsular and Schwann cells as well as nerve cells. Sharma postulated a role for ChE similar to that of AChE in the cells of the rat sympathetic ganglion cells where the distribution of the enzymes was very similar (269). In fact, ChE has been identified in gliomas (245) and glial tissue of ganglia (238).

Large concentrations of ChE were found in sensory ganglia, as well as AChE (256). Cauna (251) found ChE in many sensory receptors, particularly the touch corpuscles of human fingers and ChE was found in virtually all organized sensory end organs of skin (252). Large concentrations of ChE were found around non-myelinated nerve endings of the Pacinian corpuscle (253), although its substrate and function in these sensory receptors is unknown.

(ii) Non-Nervous Tissues

Electron-microscopic localization of these enzymes has been carried out in striated muscle (270,271,272). In 1947 Couteaux (492) suggested that the AChE in striated muscle was located in the subneural apparatus of the neuromuscular junction, all animals studied having AChE at the junction (273). Controversy over the localization of the enzymes in the myoneural junction has probably been due to diffusion of the precipitation products of the histochemical methods used (274). The locations proposed were:

(i) the plasma membrane of the muscle (271,277),
(ii) the primary and secondary synaptic clefts (271,276,277),
(iii) the plasma membranes of the axon terminal (271),
(iv) the synaptic vesicles of the axon terminal (271).

Using electron-microscopic radioautography, Salpeter (277) studied the distribution of AChE in motor end-plates of a vertebrate twitch muscle. He found 85% of the AChE localized in the junctional fold region and less than 10% in the nerve end bulb.

AChE was also found in the electric organs of fish which were found to be homologous with the motor end-plates both structurally and genetically (273), where the enzyme was essential for the production of electrical current, and the voltage per cm was directly proportional to the concentration of AChE (222).

AChE was also found at the myotendinous junction, in certain snake venoms, in platelets, and in erythrocytes, where it is bound to the outer membrane (33).

The same kind of enzyme was found in high concentrations in vertebrate diaphragm (270,243) and skeletal muscle (243,278), in adrenal gland (243), stomach (243) and placenta (279). It was also found in certain plasmas, such as those of guinea pig and rat (33).

Lewis and Shute showed that ChE was present (in addition to AChE) in the primary and secondary synaptic clefts of the rat neuromuscular junction (276). Haeggqvist (280) suggested that the enzyme in the end-plates of tonic muscles might be ChE, instead of the AChE of tetanic junctions but this remains unsupported.

ChE occurred in plasma (33,280,284), liver (281,284, 243), lung (243), pancreas (38) and other glands (243), and in intestinal muscle and mucosa (243,281,282). Studies have shown that ChE is the predominant cholinester hydrolase in the intestine, being distributed throughout the muscle layer and in both glial and ganglion cells, especially of Auerbach's plexus (238). This enzyme was also found in that part of the heart (243,281) where the rhythmic impulses regulating the automatic activity of the heart are generated (283), in the Harderian gland (243), in blood vessels (285),skin (243,281), ovary, stomach (243), in fact, in all tissues except the diaphragm (243). According to Ord and Thompson (243),those tissues in rat which received only post-ganglionic autonomic innervation contained a preponderance of ChE.

4. Physiological Functions of the Cholinester Hydrolases

(i) Acetylcholinesterase

Is the presence of AChE essential for the normal sequence of transmission under physiological conditions? Several investigators have questioned this requirement, suggesting that diffusion alone would be adequate to account for the removal of ACh, both in ganglia (327,238) and in the motor end-plate (329). The AChE, then, might possibly play a role in ACh hydrolysis when it accumulated, only during rapid neuronal activity (330).

According to the classical concept in which ACh served as the neurohumoral transmitter at synaptic and neuroeffector junctions, AChE acted to remove the ACh and terminate its depolarizing activity.

Koelle (337), however, showed a nearly exclusively presynaptic localization of AChE in sympathetic ganglia, a predominantly postsynaptic localization in the myoneural junction, and varying concentrations of AChE in noncholinergic neurones. The presynaptic nature of AChE in autonomic ganglia led Koelle and Koelle (254) to postulate that the role of AChE was to prevent postsynaptic activation by ACh released spontaneously from the preganglionic nerve endings during the resting stage (194) and to protect the presynaptic neurones from antidromic firing by released ACh (331, 332). Koelle also considered that there was no reason not to believe that the high concentration of ChE in autonomic ganglia fulfilled functions essentially similar to those of AChE (254).

Nachmansohn (200) has presented an hypothesis of a universal role of ACh in transmission, that is, that ACh also is the triggering agent for the propagation of impulses in nerve axons and muscle fibres (337). His proposal that AChE was involved in conduction along the nerve fibre (335) was based on his findings that AChE occurred in all nerves in concentrations calculated to be adequate to maintain conduction. The major objection to this theory was the extremely high concentrations of cholinester hydrolase inhibitors and blocking agents required to modify conduction in the axon, but this has been explained by the presence of structural permeability barriers shown to prevent quaternary nitrogen compounds from penetrating to the axonal membrane (222,340).

Many suggestions have been made concerning the possibility that the cholinester hydrolases might function in processes other than nervous transmission. Such processes include the regulation of local hormones, choline metabolism, ACh synthesis, aliphatic ester metabolism and transfer of acetyl groups (35).

Another interesting suggestion was that AChE might function as an agent of ACh synthesis in vivo, supplementing choline acetylase (ChA) activity (292). The concentration of AChE, substrates and the pH prevailing at the membrane, where AChE is localized (246), are not known, but for a local concentration of choline of 0.01M and a similar concentration of acetic acid, the determined association constant of 0.2 (292) would lead to an ACh concentration of 0.06 µg/ml.

A number of authors (326,181) have suggested that the function of AChE is associated with permeability processes. Rothenberg (323) reported that AChE inhibition increased the overall permeability of squid giant axons to Na^+ and K^+, and Lindvig et al. (324) demonstrated a requirement for adequate AChE to prevent loss of K^+ from the erythrocyte. The function of the high concentration of AChE on the outside of the erythrocyte membrane is unknown (308) although the proposal concerning its role in regulating permeability and active transport (308,337) received some support. Further work has not supported the hypothesis (325). The concept that ACh might play a significant role in the CNS was suggested by Dale (293) and it received some indirect experimental support (294,295). AChE of the CNS, however, had to be extensively (approximately 80%) inhibited before any effects were observed (333,334). Recently it was found that intracranial injection of cholinester hydrolase inhibitors led to temporary amnesia in trained rats which led to the suggestion that one or both of the enzymes might play a role in the learning process (300,301). The general functions of AChE in the CNS are not finally worked out.

The role of AChE in adrenergic neurones is unknown. Burn and Rand (289,290) proposed that a cholinergic mechanism was involved in the release of noradrenaline by adrenergic fibres. They concluded that adrenergic nerve impulses liberated ACh at the nerve terminals and that this

ACh released, in turn, noradrenaline. This theory would explain the weak AChE activity detected in sympathetic ganglion cells and has received much support (310,311). In line with this cholinergic link hypothesis (312,313, 314,315) was the proposed dual role of ACh of Koelle (286,337), that is, an immediate action, at the axon terminal from which it is released, which promotes the release of either enough additional ACh to act postsynaptically or another transmitter such as noradrenaline.

It has also been proposed that there might be an unknown substrate for AChE in adrenergic nerves, which might not be associated with conduction of nervous impulses (291).

The possibility that AChE might be the actual receptor substance in synaptic transmission has been advanced (341,342,343,344,345), although this would be difficult to reconcile with the presynaptic location of AChE in autonomic ganglia (254).

Finally, Koelle (239) has proposed that the presence of AChE in certain neurones may simply be a "biochemical vestige" of the phylogenetically older cholinergic neurones which have lost their ChA.

(ii) Cholinesterase

Neither the nature of the substrate nor the function of the ChE's are known. Several researchers have postulated that ChE has no important function *in vivo* (302,303,304) largely because the complete inhibition of plasma ChE was found to be compatible with life in normal circumstances, although possibly not in conditions of stress (281). The ChE of pancreas and the superior cervical ganglion could be inhibited approximately 90% without the appearance of any symptoms.

The functions of the extraneuronal ChE's in the CNS are also unknown (263). Capillaries in the CNS were found to exhibit a very strong ChE activity and it was suggested that it had a role in the function or maintenance of the blood-brain barrier (305,263,320). The significance of the strong ChE activity in glial cells is not clear (286). Koelle (287) suggested it might play some role in hydrolyzing ACh released at associated neuronal synapses, although ChE has little ACh-hydrolyzing action at low concentrations of ACh assumed to exist physiologically (297, 298). Bennett et al. have presented evidence for increased glial cells in rat cortex resulting from training (316) and the ChE of human brain appears to be particularly sensitive to inhibition by lysergic acid diethylamide whereas AChE is unaffected (346), indicating a possible role in mental processes.

Earl and Thompson (40,319) suggested that extraneuronal ChE might be concerned in myelin metabolism and the maintenance of the myelin sheath because some of its inhibitors caused demyelination and paralysis in chickens. Others, however, (317) showed that the relationship between inhibition of ChE and neuropathy and spinal tract degeneration was not direct although only those organophosphates known to be selective inhibitors of ChE produced paralysis.

ChE has been implicated in relatively slow nerve conduction processes (318), and Haeggquist(280) suggested that the enzyme in the end-plates of tonic muscle was ChE, instead of the AChE at tetanic muscle junctions. He postulated that the slower hydrolysis of the transmitter might account for the prolonged action of ACh on smooth muscle.

Hokin et al. have proposed that ACh increased the incorporation of phosphate into various phospholipids in slices of cat sympathetic ganglia (306, 307) and they also suggested a role in the regulation of phospholipid metabolism for extraneuronal ChE (321).

Burgen and MacIntosh (168) suggested that the natural substrate for ChE might be something other than ACh. Outside the nervous system ChE could, for example, hydrolyze toxic acylcholine esters such as PCh and BuCh which can be formed in fatty acid oxidation from acyl-CoA and choline (288). These compounds have been isolated from tissue extracts (48) and are known to inhibit AChE (281). Thus ChE might have a role in protecting AChE from inhibition, as Koelle suggested, a "scavenger" role (322).

There is considerable evidence that ACh acts as a local hormone, released by and acting on such muscle fibres as those of the heart and intestine (337). ACh in such tissues arises from non-nervous (i.e., muscular) sources, and regulates the tone and rhythmicity of the muscle. Jamieson (297, 336) concluded that ChE destroyed only ACh of non-nervous origin in intestine whereas AChE destroyed both neurogenic and non-nervous ACh.

5. Specificity of the Cholinester Hydrolases

The substrate specificities of the cholinester hydrolases have been reviewed by Whittaker (35), Barlow (167), Nachmansohn and Wilson (335), Augustinsson (33,28) and several others.

The classification of these esterases as AChE and ChE does not necessarily imply that the components of each group have identical properties. Evidence, in fact, has indicated differing sensitivity to inhibitors of the enzymes belonging to the same group (347). Chicken ChE was found to be insensitive to many of the specific inhibitors used for both AChE and ChE (348).

Considerable evidence has been found that relatively slight modifications in the chemical structure could influence the hydrolysis of substrates (349) or the strength of cholinester hydrolase inhibitors (350). In such studies, however, it is important to recall the variation in specificity of the enzymes of different sources. The AChE of the electric organ of *Electrophorus electricus* had the same optimum concentration for PCh as for ACh so that either ester could equally well be the natural substrate for the enzyme, while erythrocyte AChE hydrolyzed ACh more rapidly than PCh (58).

There was marked variation in the cholinester hydrolase activity of normal human subjects, particularly of human plasma ChE and the esterase values appeared to vary with time even in the same individual (351).

The substrate specificity of the enzymes from the same tissue in different species varied. The ratio of hydrolysis of SDCh to ACh was, for example, higher in human serum than in dog serum (352) and a species variation in the ratio of hydrolysis rates of ACh to BzCh by sera was also found (353).

(i) Effect of Variations in the Acyl Group

Generally, the maximum rate of certain enzyme reactions has been found to increase as much as two to three times with a single additional carbon atom in a "chemically inert hydrophobic region of the substrate molecule" (354). The "optimum" chain length was determined by the acyl group of the ester substrates and was specific for each esterase tested. This relationship held true for the cholinester hydrolases (354).

Adams and Whittaker (355) found that AChE hydrolyzed acetate esters more rapidly than formate or propionate esters and had little action on butyrate esters. That is, the rate of hydrolysis decreased with increasing length of the aliphatic chain of simple choline esters (18) up to laurylcholine (356). This same specificity was exhibited by ChE when the aliphatic acid was longer than butyric acid (18). ChE hydrolyzed BuCh very rapidly, approximately two to three times the rate of ACh (35). Long chain fatty acid esters such as palmitoylcholine were not hydrolyzed by either enzyme (18).

Not one of 17 ω-amino substituted fatty acid esters of choline was hydrolyzed by AChE (357). Neither the nonsubstituted nor the ω-amino-substituted choline esters inhibited the hydrolysis of ACh by ChE whereas the inhibitory effects of both series of compounds on AChE increased with increasing values of n in the fatty acid chain. Some of the monomethyl- and dimethylamino and trimethylammonium derivatives, on the other hand, inhibited ACh hydrolysis by both enzymes (357). Particularly interesting was the finding that ChE did not hydrolyze 4-trimethylammonium butyrylcholine (GBB choline) (357).

Separation of the substituted nitrogen group from the ester bond, as in acetyl-γ-homocholine, decreased enzymic activity (18).

The carbamic ester of choline, carbaminoylcholine, was not hydrolyzed by either enzyme, being a weak inhibitor of both (358,359).

Unsaturated aliphatic acid esters of choline were found to be very poorly hydrolyzed by AChE, although several were good substrates for ChE (360, 361,362). Crotonylcholine was a weak inhibitor of AChE and its ß-methyl derivative was an inhibitor of ChE (360,361,362).

Aromatic choline esters were generally not hydrolyzed by AChE, but were substrates for the ChE's (363). Replacement of the ether oxygen by a sulphur atom, however, decreased hydrolytic rates (322).

Koelle (322) found that the thioesters of ACh and acetyl-ß-methylcholine were hydrolyzed more rapidly than their oxygen analogues by both types of enzyme. In recent studies on a series of sulphur and selenium isologues of ACh (212,209) which were isosteric, hydrolysis rates by AChE were very similar.

Very early studies showed that (±)-acetyl-α-methylcholine was hydrolyzed at 70% the rate of ACh by horse serum ChE, whereas (±)-acetyl-ß-methylcholine was hydrolyzed only at 2% the rate. With the α-methyl derivative no stereospecificity was noted, whereas only the (+)-acetyl-ß-methylcholine isomer was hydrolyzed (364).

Chloroacetates and bromoacetates were hydrolyzed very rapidly in comparison with aliphatic esters, exceeding even the rates for choline esters by AChE (355).

(ii) Effect of Variations in the Choline Moiety

The specificities of AChE and ChE were primarily determined not by the chemical nature of the substrate molecule, but by its shape according to Adams and Whittaker (355,365,366,367).

Glick (19) studied acetyl esters of structures analogous to ACh. He demonstrated a requirement for a configuration closely similar to that of the choline ester for hydrolysis by ChE. 2-Acetoxymethyl (dimethylpyrrolidinium) and 2-acetoxymethyl (dimethylpiperidinium) were hydrolyzed rapidly while the isomeric 3-acetoxymethyl (dimethylpiperidinium) was a very poor substrate.

Strack _et al._ (368) demonstrated that acetylcarnitine (AC) was not hydrolyzed by AChE unless the carboxyl group was esterified. Later Fellman and Fujita (369) proposed that this might be explained by the neutralization of the quaternary nitrogen positive charge by ring formation.

(iii) Effect of Variations in the Onium Group

Holton and Ing studied the specificity of the trimethylammonium group in ACh (370). The mono-, di-, and tri-N-ethyl analogues of ACh were hydrolyzed by the AChE of dog caudate nucleus and cobra venom at approximately the same rate as ACh. The rate of hydrolysis by horse serum ChE declined with successive substitution of the methyl by ethyl groups. Both ester groups of bis-acetoxyethyldimethylammonium iodide were hydrolyzed by both AChE and ChE.

ACh, acetylphosphocholine, and acetylarsenocholine were all hydrolyzed at similar rates by both enzymes (371).

The carbon analogue of ACh, 3:3-dimethylbutylacetate, was hydrolyzed by human erythrocyte AChE at 60% the rate of ACh. The same relation held true for BuCh and its aliphatic butyrate analogue (355, 365,366,367).

1-(2-Acetoxyethyl)-quinuclidinium iodide was not a substrate for either AChE or ChE, being a competitive inhibitor of AChE (372). It was concluded that the rigid conformation rather than the overall size of the onium group was critical in preventing acetylation of the enzyme.

(iv) Bis-onium Compounds

Barlow (373) studied a series of polymethylene bis-acetoxyethyldimethylammonium salts and found that the rates of hydrolysis by both AChE and ChE varied with the length of the aliphatic chain. For AChE there was an optimum hydrolytic rate with the octamethylene compound. Although for the serum ChE all compounds having more than a 4 carbon chain were excellent substrates themselves, they were inhibitors of ACh hydrolysis. The hydrolysis of SDCh, another bis-quaternary compound, was caused by horse serum ChE (20), human serum ChE (374), and both AChE and ChE of rabbit blood (375). The hydrolysis of ACh by both enzymes was inhibited by SDCh (100). There was also rapid hydrolysis of higher dicarboxylic acid esters of choline by ChE (e.g., sebacinyldicholine, suberyldicholine) (352,376,377).

6. Stereospecificity of the Cholinester Hydrolases

The stereochemical specificity of AChE with respect to substrates with asymmetric carbons in the acid group of choline esters has received little experimental attention. Serum ChE, however, was found to hydrolyze the D-(-)-isomer of mandeloylcholine faster than the L(+)-isomer (378).

L(+)-Lactoylcholine was hydrolyzed far more rapidly than D(-)-lactoylcholine (LCh) by electric eel AChE (379). At high substrate concentrations

L(+)-LCh was hydrolyzed more rapidly than ACh and at approximately the same rate at low substrate concentration. D(-)-LCh was found to inhibit hydrolysis of L(+)-LCh (379) which itself exhibited substrate inhibition (380). The carbonyl carbon of LCh was more electrophilic than that of ACh and thus might be more susceptible to hydrolysis by the nucleophilic group of the esteratic centre (379).

The levo isomers of α-amino acids were better inhibitors of AChE than their corresponding dextroisomers (381).

AChE from several tissues hydrolyzed only the dextro (+) isomer of acetyl-ß-methylcholine (18,382,383) whereas the acetyl-(L)-ß-methylcholine isomer was an inhibitor of the hydrolysis of the D isomer and of ACh (384).

Although L-acetyl-ß-methylcholine (Ac-ß-MeCh) was only a weak inhibitor the effect was emphasized by Hoskin (383) because, in the generally available racemic mixture, the initial concentrations of inhibitor and substrate for the enzyme are equal.

From the stereospecificity of the hydrolysis of acetyl-ß-methylcholine, and the lack of stereospecificity for the hydrolysis of acetyl-α-methylcholine, it was concluded that the ß-methyl group interfered with the binding of the ester group to the enzyme, in the L-isomer. Ellenbroek and van Rossum (385) showed that the (+) isomer, the substrate for AChE, had the S-configuration.

According to Ooms and Boter (386) AChE was stereospecific for several series of optically active organophosphorus inhibitors whereas ChE was much less stereospecific.

The O-C-C-N^+ system is a structural feature of phospholipids and many ACh analogues and it was found to exist in the *gauche* form, rather than the *trans* form which would have been expected on purely steric grounds (387). Sundaralingam (387) used this close structural relationship between phospholipid components and ACh to suggest that the lecithin and sphingomyelin of cell membranes might be both competitive inhibitors and sites of concentration of AChE (387). Studies of ACh analogues containing the O-C-C-N^+ backbone of ACh in rigid conformations showed that the *trans* conformation of ACh was congruent with the ACh receptors in smooth and skeletal muscle, but not with the ganglionic receptors, while the opposite was true of the *cis* conformation of ACh (388).

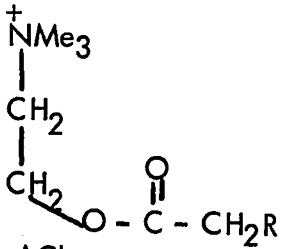
The enzymatic discrimination between enantiomorphs is usually explained by proposing the existence of an asymmetric reactive site on the enzyme surface. This site would then facilitate the hydrolysis of one stereoisomer in preference to another in view of a three point combination of groups attached to an asymmetric carbon of the substrate with three sites on the enzyme surface.

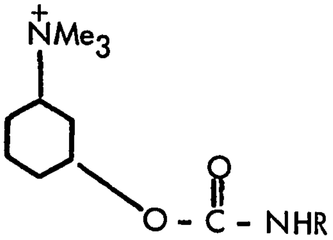
7. Inhibition of the Cholinester Hydrolases

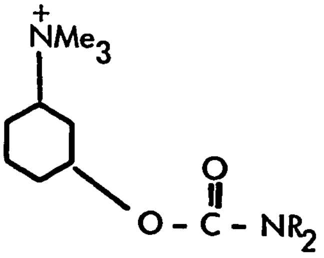
A distinction between nonspecific esterases and the cholinester hydrolases could be drawn on the basis of their susceptibility to eserine. The cholinester hydrolases were completely inhibited by 10^{-6}M eserine (389,26), while the nonspecific esterases, hydrolyzing short-chain fatty acid esters, were only slightly affected by 10^{-3}M eserine.

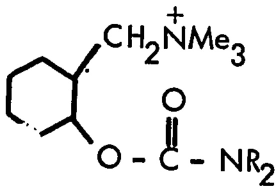
Although in older work the esterases were characterized by the use of different substrates, Aldridge and others (390,273,391) pointed out the usefulness of employing a variety of selective inhibitors for this purpose. Bayliss and Todrick (392) used specific inhibitors of AChE in the estimation of ChE in nervous tissue, and Hawkins and Mendel (393,37) demonstrated the relatively specific inhibition of ChE by low concentrations of diisopropyl fluorophosphonate (DFP).

The specific anti-"cholinesterases" have been extensively reviewed (394). Many exhibit a closely analogous configuration to that of ACh, supporting Fischer's (395) "lock and key" theory. The following structures of specific inhibitors indicate the complementary relationship between ACh and certain competitive inhibitors.


ACh


miotin (396,397)


prostigmine (397)



Nu-series (398)

Values obtained for cholinester hydrolase inhibition were found to vary according to the arbitrary conditions of the experiments. An example quoted by Burgen (399) was the determination of the inhibitory activity of eserine. Acetyl-ß-methylcholine, a specific substrate for AChE, had a lower affinity for the enzyme than ACh and therefore, when it was used as a substrate, eserine showed a greater degree of AChE inhibition than it did with ACh. Also it made a difference when the inhibitor was added. For eserine, equilibrium inhibition was determined competitively by the concentrations of substrate and the inhibitor (400), a competitive inhibitor.

Eel AChE and horse serum ChE were recently found to be appreciably inhibited by Tris buffer in the concentrations usually used in buffer solutions. Tris reacted with the esteratic site of both AChE and ChE, inhibiting both enzymes competitively even at 1 mM (401). The inhibition constants for the reaction between Tris and AChE and ChE respectively, with ACh and BuCh as substrates, were reported to be the same: 13-14 mM. This should be remembered as Tris is used as the buffer in many standard cholinester hydrolase determinations.

Choline was an inhibitor of AChE, chiefly due to product inhibition although competition for the enzyme anionic site also caused some competitive inhibition (402). Other naturally-occurring cholinester hydrolase inhibitors were found in animal tissues (403). For example, BuCh strongly inhibited the hydrolysis of acetyl-ß-methylcholine (MeCh) by erythrocyte AChE, while MeCh slightly inhibited BuCh hydrolysis by ChE (404).

Phosphorylcholine was a strong inhibitor of AChE (405,387) and Holmstedt (337) studied the quaternary alkylphosphate anti-"ChE's", all of which were potent inhibitors and had the added characteristic of being choline esters. As such, they are potent tools for the study of these esterases.

8. Multiple Forms of Cholinester Hydrolases

Electrophoretic, chromatographic and kinetic studies indicated that human and horse sera contained more than one ChE with only slightly different properties (286). In human liver three enzymatically identical ChE's could be separated by electrophoresis and chromatography (286). Comparison of the rates of hydrolysis of ACh, BzCh, SDCh, and procaine by human serum indicated also the presence of more than one esterase hydrolyzing ACh (406) and Holmes and Masters found at least 5 isoenzymes of cavian ChE's (407).

Bernsohn et al. discovered multiple forms of AChE in nerve tissue (408) and several protein fractions possessing AChE activity, including AChE, myosin, and actomyosin, were isolated from rabbit striated muscle (409). In zymograms 3 AChE fractions could be observed with acetylthiocholine and α-naphthyl acetate as substrates (286).

Three species of AChE sedimenting at different rates were reported by Grafius and Millar (410). Evidence for a very high molecular weight species of highly purified AChE had been previously reported by Rothenberg and Nachmansohn (411) and by Lawler (412). Grafius and Millar suggested that AChE might exist in an aggregated or polymeric form in the tissue and that it might be converted to smaller units by treatments during extraction, depending largely upon the ionic strength of the medium (410). It was subsequently suggested (413) that such a salt- and pH-dependent reversible aggregation of AChE might play a role in the control of the permeability changes involved in neuronal behaviour.

9. Properties of the Cholinester Hydrolases

(i) Acetylcholinesterase

The enzyme from the electric organ of *Electrophorus electricus* has been purified by ammonium sulphate precipitation and high-speed centrifugation to yield a preparation with a specific activity of 400 mM ACh/mg dry wt/hr (414) and purified preparations have been obtained from human and ox erythrocytes (415,416). Lawler has purified AChE from dog brain and found the solubilized purified preparations to be very unstable (417) unlike the stable preparation from the electric tissue of eel. The particulate nature of AChE has made it difficult to obtain the enzyme pure in solution although its solubility in salt solutions and in the presence of surface active agents and solubilizing enzymes has been intensively investigated (417,229, 44). Leuzinger and Baker carried out large-scale purification of the electric organ AChE in 1967 making possible the study of the protein properties of the enzyme (418).

The pH optimum of AChE's was found to be in the range 7.5 to 8.5 (33).

The enzyme exhibited substrate inhibition (419). Certain alcohols were found to activate AChE (420), e.g., water-miscible organic solvents caused activation of honeybee head AChE (421).

Purified rat brain AChE was less soluble in 0.1 to 0.2 M NaCl and sodium phosphate buffers than in distilled water (417). Although there was no evidence that metals were required for AChE activity, salts such as NaCl, KCl, and $CaCl_2$ activated AChE and raised the optimum substrate concentration (422). Studies showed that the anion associated with the substrate (chloride, bromide, iodide, or perchlorate) had no effect on the rate of enzymatic hydrolysis by AChE (18).

Recent studies showed that membrane-bound AChE behaved kinetically, at an ionic strength of 0.15, as if it were soluble. The K_m for the soluble AChE was reported as 79 μM, and that for the membrane-bound AChE as 84 μM (418, 423). Earlier work had shown that the K_m of AChE decreased very rapidly with decreasing ionic strength (424). Most reports of the Michaelis-Menten constant for AChE give values between 10^{-3} and 10^{-4}M. Values of 2.6×10^{-4}M and 0.94×10^{-4}M at pH 7.0 for the K_m of eel AChE have been reported (425,426,427).

With AChE spread on the surface of a solution, Skou (428) demonstrated the great influence of pressure upon enzymatic activity. He concluded that proper orientation and folding of the protein had an essential role in enzymatic activity.

According to Nachmansohn and Wilson (335) and Rothenberg and Nachmansohn (411), 1 mg of a highly purified eel electric organ AChE preparation split 75 mg ACh/hr at 37°C and 0.018M ACh. A molecular weight of 3,000,000 was reported for the preparation (411), giving a turnover of 20,000,000 molecules ACh/min/molecule AChE (335). Studies by Michel and Krop (429) showed that this molecular weight represented 48 active centres which could be explained as an aggregation of smaller molecules of molecular weight 63,000. Using their figure of 48 active centres, they calculated a turnover number of 438,000 molecules ACh hydrolyzed/active centre/min for electric eel AChE. A turnover number of 295,000 was found for ox erythrocyte AChE (430).

Serlin and Fluke (431) found a molecular weight of 105,000 for their AChE preparation. As discussed in Section A.8, this discrepancy may be due to differences in extraction procedures, or the existence of multiple enzymes. A study of the interaction of pH and ionic strength on reversible aggregation of eel AChE demonstrated a major effect of ionic strength on molecular weights obtained by centrifugation (413).

For the reaction

$$\text{ACh} + H_2O \xrightarrow{\text{ChE}} \text{Acetate} + \text{Choline}$$

Hestrin (292) found a Nernst constant of 0.25 at 23°C and the free energy of the reaction was calculated to be 3,200 cal.

An activation energy of 3,700 cal/mole was reported for human erythrocyte AChE with ACh as substrate (432) although the energy of activation of this reaction was later found to vary with temperature, giving curved Arrhenius plots (433).

The hydrolysis of ACh was found to occur in under 0.1 msec (412,429,434), occurring in as little as 3 to 4 μsec (222).

(ii) Cholinesterase

The molecular properties of the non-specific ChE's have been reviewed in detail by Svensmark (435).

The enzyme has never been completely purified. Good preparations include (a) precipitation of the ChE of dog pancreas by ammonium sulphate and adsorption on kieselguhr (38), (b) precipitation of horse serum ChE by ammonium sulphate (436), and (c) ethanol precipitation of human serum ChE (437). Serum ChE's from human and horse were soluble in water, precipitated by 2 to 3 M ammonium sulphate and soluble in the presence of zinc ions (435).

The serum enzyme was found to be produced in the liver (438,439) and was apparently identical with the procaine esterase found in human plasma (440). Plasma ChE was composed of a group of related glycoproteins and represented only 0.01% of the total plasma protein (441). Human serum ChE, composed of one of two types of enzyme (442), was an α_2-globulin sialoprotein (441).

There was no evidence of a requirement for metals by ChE's and treatment of human serum ChE with neuraminidase removed several sialic acid moieties, not affecting the hydrolysis by the enzyme (435).

Activation of serum ChE by Ca^{++} and Mg^{++} ions was a specific effect, not related to the ionic strength (443). Although it was suggested that these ions were required (443) for enzymic function, this was not supported by Svensmark (435). The rate of hydrolysis of BzCh by ChE was found to vary markedly with alteration of the ionic strength (445) and large dilutions inactivated serum ChE (444) as did drying with acetone (444).

The ChE's isolated from several tissues differed with respect to heat sensitivity (409) but the optimum temperature of horse serum ChE was 45°C (446).

In determining pH dependence, ChE was found to be independent of the type of buffer used and had a pH optimum range of 7.5

to 8.5 (446,33).

ChE was not subject to substrate inhibition with ACh, PCh, or BuCh as substrate (33).

The molecular weight of human serum ChE was estimated at approximately 300,000 (435) and for horse serum, which may have 2 to 4 active sites, the molecular weight (per active centre) was calculated to be 84,000 (435).

For the ChE's of serum the values of K_m generally have been reported to lie between 10^{-2} and 10^{-3} M (167), for example, $10^{-2.5}$M for human serum ChE with ACh (419), and $10^{-2.8}$M for human serum ChE with BuCh (360), 2 x 10^{-5}M for horse serum with BzCh and 1 x 10^{-3}M for the horse serum ChE with ACh (446).

The turnover number for horse serum ChE was 50,000 molecules ACh hydrolyzed/active centre/minute (448).

The activation energy for human serum ChE was 7,700 cal/mole (432) and for horse serum ChE, 5,100 cal/mole (447).

10. Evidence for Substrate Attachment at Two Sites on the Enzyme

Much of the information about the nature of the active centres of the cholinester hydrolases has been obtained by consideration of the chemical structures of substances which have an affinity for the enzymes and the effects of pH on the activity of the enzymes and substrates.

The term "active site" or "active centre" refers to those regions of the enzyme surface where the substrate is localized and activated during enzymic action.

When the concentration of AChE is of the order of 0.001%, and that of ACh is below 3 mM, the system exhibits Michaelis-Menten kinetics but the velocity of the reaction decreases at higher substrate concentration. This marked substrate inhibition was first studied by Murray (449) and Haldane (450). Murray suggested that the inhibition might be explained if one ester molecule were attached to the enzyme by the acyl group and a second substrate molecule by the alcohol group at a two point active site. Zeller and Bisseger (32) also suggested that ACh might be attached to AChE at two points. The substrate inhibition could arise from the adsorption of two separate ACh molecules at these sites simultaneously. Since the two functional groups on the ACh molecule involved in binding were thought to be the quaternary nitrogen group and the ester group (355,425,381,451), the sites in the active centre were designated the "anionic" and "esteratic" sites, respectively.

$$E + S \overset{K_1}{\rightleftharpoons} ES \xrightarrow{k} E + P$$

$$ES + S \overset{K_2}{\rightleftharpoons} ES_2$$

where K_1 and K_2 are dissociation constants, k is the velocity constant.

Humped initial velocity-substrate concentration curves were not found for the hydrolysis of ACh and BuCh by plasma ChE (33). For this reason and from the studies of Adams and Whittaker (355) on the relative affinities of AChE and ChE for ACh and its carbon analogue, it was concluded that ES_2 formation might not be significant in ChE due to the lack of an "anionic" group.

Specificity studies with AChE which showed that specificity was determined by both parts of the ester substrate molecule supported the concept of a two-point attachment. A more recent explanation for the marked substrate inhibition was suggested on the basis of a two-step hydrolytic process which is discussed below. In such a mechanism the acetyl-enzyme might bind a second ACh molecule at the "anionic" site and this could then inhibit the hydrolytic deacetylation of the enzyme (427).

(i) The Anionic Site

In 1948 Augustinsson showed that the affinity of AChE for ACh was decreased by the addition of the positively charged protein, clupein, while the negatively charged gum arabic had the reverse effect (452). He concluded that the affinity for ACh might be determined by the total negative charge on the enzyme. He found no such effect on ACh-ChE interactions.

The ratios observed, however, for (i) the affinities of choline for the AChE and ChE of human blood (453), and (ii) the affinities of ACh and its uncharged carbon analogue for ChE (355) were consistent with a single negatively charged atom in the active centre (424) of AChE which was absent in that of ChE (355). It appeared that this might be involved primarily in anchoring molecules to the active centre. For example, the competitive inhibition of AChE by simple quaternary ammonium salts was explained solely by their affinity for the "anionic" site, coupled with unspecific van der Waal's forces binding the aliphatic groups to the enzyme surface (454).

Studies by Bergmann and Segal in 1954 showed the presence of an "anionic" site near the esteratic site of serum ChE, indicating that the active centres of ChE and AChE might be essentially similar (318). Further investigations with bis-quaternary compounds, however, demonstrated that AChE possessed two "anionic" sites, binding both quaternary ammonium groups while ChE contained only a single "anionic" site (455,456). The possibility that a single molecule of ACh or inhibitor could be attached simultaneously to both "anionic" sites of AChE was discussed (456). The variable influence of chain length and the inhibitory effect of ω-amino fatty acid esters of choline which had two positively charged nitrogens supported the concept that, while ChE had one, AChE had two closely placed "anionic" sites (357). Although many long chain bis-onium salts possess very high affinity for AChE's, the possibility exists that long polymethylene bis-quaternary salts might owe this high affinity in large part to van der Waal's adsorption along the chain in addition to binding to the "anionic" and "esteratic" sites (430).

Augustinsson has found that ChE contained a second "non-esteratic" site which differs from the "anionic" site of AChE. The dominant force involved in reactions with this second site of ChE were van der Waal's forces, unlike the Coulombic attractions which favour complex formation with the "anionic" site of AChE (457).

Because affinity is not the only factor influencing the rate of hydrolysis, the anionic site does not appear to be essential for interaction with the substrates. Adams and Whittaker (355), for example, found that 3:3-dimethylbutylacetate was hydrolyzed at 60% the rate of ACh by AChE. Comparing ACh and its tertiary analogue, dimethylaminoethylacetate, the cationic species was much more readily hydrolyzed (424), although at pH 7 the base itself was definitely hydrolyzed (425). The inhibition of eel AChE by neostigmine was unaffected by pH, whereas the effects of eserine declined in alkali (425), i.e., the cation was more active than the free base, eserine. Wilson and Bergmann (425) calculated that conversion from the free base to the cations increased the affinity of the inhibitors for the enzyme twenty-fold.

Zupancic (458) found an equilibrium constant for the interaction of ACh and the horse serum ChE anionic centre to be in the range from 10^{-7} to 10^{-6}M.

It has been assumed that the anionic group on the enzyme interacts with the outer surface of the cationic group (377). The nine hydrogen atoms of the trimethylammonium group lie on a spherical surface with a radius of approximately 2Å, the surface possessing a positive electrical charge (459). Replacing the trimethyl- with a triethylammonium group increases the radius of the head to about 3.2Å but has little effect on the hydrolysis of ACh by AChE while greatly reducing the activity of ChE (370). Studies by Wilson (424) have demonstrated that whether one has the full complement of methyl groups or whether one group is removed makes little, if any, difference in the binding of the substrate to the enzyme.

(ii) The Esteratic Site

The hydrolysis of ACh by electric eel AChE was found to be pH dependent, with the maximum hydrolytic rates in the pH range 7 to 9 (425). Wilson and Bergmann (425,426) suggested that the active centre was amphoteric and that the species (EH) containing undissociated acidic and free basic groups was most active.

$$EH_2^+ \rightleftharpoons EH \rightleftharpoons E^-$$

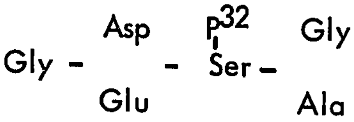
They suggested that the nucleophilic group formed a link with the electrophilic carbon of the substrate acyl moiety, while the acidic group formed a link with one of the ester oxygens, leading to the electronic shifts which activated the ester link (35). There appeared to be a correlation between the affinity of substances for the esteratic site and the electrophilic nature of their carbonyl carbons (35).

The dissociation constants of the nucleophilic (pK_a=6.5) (460,461,462) and the electrophilic (pK_b=9.3) groups of the esteratic site were calculated by Bergmann et al. (460). The value of pK_a suggested the presence of a histidine residue (460,463,464,465) in the active site. The value of pK_b appeared to be due to the p-hydroxyl group of a tyrosine residue (425,426,460).

The change in rate of hydrolysis with pH was studied for a variety of esterases using non-cationic substrates (460). The esteratic sites of all the different esterases examined possessed approximately the same dissociation constants. It was concluded, therefore, that the esteratic sites of the esterases, including ChE and AChE, were chemically closely related.

Cohen et al. (466) demonstrated that a number of esterases were sensitive to the organophosphorus inhibitor diisopropylfluorophosphonate (DFP). This compound phosphorylated the enzymes combining irreversibly with that part of the active centre concerned with activation of the ester link of the substrate and yielding a stable inactivated enzyme (467,468). Degradation of the phosphoryl enzymes yielded a common structure (called the B group) labelled with DFP (469).

In every case O-phosphoryl serine was isolated (469)- from erythrocyte AChE (448), eel AChE (448), serum ChE (448), and horse serum aliesterase (469). In addition to the amino acid histidine, group B occurred in all esterase active sites (469).



$$\text{Gly}-\begin{matrix}\text{Asp}\\ \\ \text{Glu}\end{matrix}-\overset{\overset{\text{P}^{32}}{|}}{\text{Ser}}-\begin{matrix}\text{Gly}\\ \\ \text{Ala}\end{matrix}$$

"Group B" (469)

Cohen et al. (466) suggested that this structure was in some way responsible for general enzymatic ester hydrolysis whereas particular substrate specificity would be determined by other groups near the esteratic site.

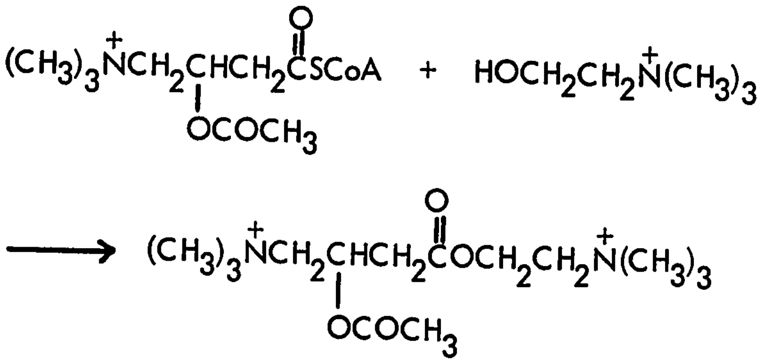
The inhibition of AChE by a number of compounds has also been related to molecular complementariness by Wilson and Quan (349). They demonstrated that the atom of AChE which formed a covalent bond with the carbonyl carbon atom of a carbamate inhibitor was the basic group of the esteratic site, which was situated at about 5 Å from the anionic site.

C. Acetylcarnitylcholine

The presence of l-carnitine in tissues has been established, although its function in nerve and muscle is uncertain. Acetyl-l-carnitine (AC) possessed only weak cholinergic activity (134,493), although esterification of a carboxyl ester derivative of carnitine gave increased cholinergic activity (140).

1. Synthesis of Acetyl-l-Carnitylcholine

Hosein and Koh (139,136) reported that acetyl-l-carnityl CoA was the major cholinergic material which accumulated in the brains of narcotized rats. The synthesis of acetyl-l-carnitylcholine (ACCh) was regarded as a reaction involving a butyryl CoA derivative (acetyl-l-carnityl CoA is γ-trimethylammonium-β-O-acetylbutyryl CoA). Subsequently Hosein *et al.* (138) presented evidence that ACCh could be synthesized *in vitro* by the esterification of choline by acetyl-l-carnityl CoA from narcotized rat brain extracts in the presence of the non-specific enzyme, choline acetyltransferase (E.C.2.3.1.6.) (138). More recently, the chemical synthesis of ACCh and other carnitine derivatives has been reported by Kato and Hosein (470).



$$(CH_3)_3\overset{+}{N}CH_2\underset{\displaystyle OCOCH_3}{\underset{|}{C}}HCH_2\overset{\displaystyle O}{\overset{\|}{C}}SCoA \;+\; HOCH_2CH_2\overset{+}{N}(CH_3)_3$$

$$\longrightarrow (CH_3)_3\overset{+}{N}CH_2\underset{\displaystyle OCOCH_3}{\underset{|}{C}}HCH_2\overset{\displaystyle O}{\overset{\|}{C}}OCH_2CH_2\overset{+}{N}(CH_3)_3$$

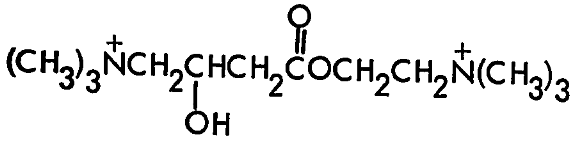
2. Properties of Acetylcarnitylcholine

ACCh, a disubstituted carnitine derivative, exhibits pharmacological properties very similar to those of ACh. ACCh is structurally "two ACh molecules", is an isomer of SDCh, and is both a choline and a carnitine ester.

The ACh-like activity of synthetic dl-ACCh was found to be one-eighth that of ACh on a molar basis on three preparations. It was equiactive on the eserine-sensitized frog rectus and guinea pig ileum preparations, giving a 1:1 nicotinic to muscarinic potency ratio (471).

dl-ACCh was four times more potent than ACh as a neuromuscular blocking agent on the rat phrenic nerve-diaphragm and frog sciatic nerve-gastrocnemius preparations (472).

The closely related compound, carnitylcholine (CCh) has



$$(CH_3)_3\overset{+}{N}CH_2\underset{OH}{C}HCH_2\overset{O}{\overset{\|}{C}}OCH_2CH_2\overset{+}{N}(CH_3)_3$$

been chemically synthesized (470) and found to be a very potent neuromuscular blocking agent (475). It appeared to be resistant to the actions of the acetylating enzymes from rabbit brain acetone powder (475) and had a more prolonged blocking action than that of ACCh (475).

3. Isolation of Acetylcarnitylcholine

l-ACCh and its precursor, acetyl-l-carnityl CoA would be expected to be present in animal tissues only in minute amounts (<1 μg/g fresh brain tissue) (138). Recently evidence based on both prolonged paper chromatography (473) and column chromatography (474) has established the presence in rat brain extracts of l-ACCh.

STATEMENT OF THE PROBLEMS STUDIED

From a review of the literature, three areas of investigation were undertaken for further study.

I. Hosein et al. (53,132,133) have shown that in rat brain extracts there are several quaternary ammonium compounds which contribute to the total ACh-like activity. Chemical examination, after chromatographic separation, demonstrated the presence of choline, ACh, PCh, BuCh and a mixture of the CoA esters of GBB, crotonbetaine, carnitine and AC (54,139). Recently the presence of a very active compound, an acetyl-l-carnitylcholine (l-ACCh), in rat brain extracts has been demonstrated by column chromatography and paper chromatography. The pharmacological properties of the extracted material and chemically synthesized l-ACCh have been investigated (471,473) and have suggested a possible neurohumoral transmitter function for this compound.

A fundamental criterion for transmitter function is that of the Inactivating Enzyme (494). In view of the extensive literature concerning the relative lack of substrate specificity and the stereospecificity of the cholinester hydrolases, the hydrolysis of dl-ACCh by these enzymes was investigated.

II. The specificity of acetylcholinesterase for acetate esters has been well documented (365,366,367). It appeared that if acetate was hydrolyzed from ACCh, the product would be carnitylcholine, a very powerful neuromuscular blocking agent (475). If, on the other hand, the choline moiety were removed, acetylcarnitine would be released. To study the possible site of enzymatic cleavage, the hydrolysis of acetylcarnitine and carnitylcholine by the cholinester hydrolases was investigated.

III. An hypothesis has been proposed that the function of cholinesterase is to protect acetylcholinesterase from inhibition by naturally-occurring inhibitors, e.g., PCh and BuCh (288). The structures of the compounds ACCh, CCh and acetylcarnitine suggested that these might also be competitive inhibitors of acetylcholinesterase. Thus, the inhibition of the enzyme by a series of choline and carnitine esters was investigated.

METHODS

Part A. Chemical Synthesis of Acetyl-dl-Carnitylcholine and dl-Carnitylcholine

Acetyl-dl-carnitylcholine (dl-ACCh) and dl-carnitylcholine (dl-CCh) were prepared in our laboratory by G. Kato (471,470). It was necessary to remove impurities from freshly prepared l-CCh before acetylation. The following steps were involved. The compound was applied as a narrow band on thin-layer plates (Silica Gel G, Analtech, 20 x 20 cm, 400 μ) and chromatographed in an ascending system of Methanol/Acetone/HCl (90/10/10, v/v) (482) for two hours. Each plate held approximately 25 mg of the compound. After drying, the appropriate area of the chromatogram (R_f 0.05 to 0.3) was scraped off the plate with a spatula, collected in a beaker and eluted by shaking in absolute methanol for two hours. The material was then filtered through Whatman No.1 paper and the methanol was evaporated, leaving behind a thick colourless viscous material.

dl-CCh, prepared in this manner, was acetylated by refluxing a mixture of dl-CCh, acetic acid and acetylchloride for three hours. The acetyl chloride and acetic anhydride were subsequently removed by flash evaporation.

1. Nuclear Magnetic Resonance (NMR) Spectra

Nuclear magnetic resonance spectra and infrared spectra were carried out by G. Kato on each new batch of material to determine their purity.

2. Thin-Layer Chromatography (TLC)

When necessary, dl-ACCh was purified by TLC repeating the method outlined for dl-CCh given above. Although losses of dl-ACCh were very high (as much as 50%) the procedure was adequate for the removal of the contaminants dl-carnitine and choline.

Part B. Separation of Choline and Carnitine Derivatives

1. Thin-Layer Chromatography (TLC)

Thin-layer chromatography of the butyrobetaines and other quaternary compounds was according to Eneroth and Lindstedt (482) as outlined in Methods, Part A.

2. Paper Electrophoresis

The method of Stavinoha and Ryan (75) was followed for the separation of choline and carnitine esters. The Whatman No. 1 paper chromatograms (57 x 46 cm) were developed in an ammonium acetate solution (0.5 M) at pH 4.6 for 1.5 hours at 1500 volts in a Gilson Model D high voltage electrophoresis apparatus. The pH was checked at the termination of the run as the choline esters were only stable in the pH range from 3 to 6.

3. Paper Chromatography

Test solutions containing 10 to 50 µg ester were applied to Whatman No.4 paper with a micropipette on a faint pencil line drawn on the paper parallel to and 10.0 cm from one end. Ascending chromatography was carried out after the method of Davies and Kalow (108). The chromatogram was fashioned into a cylinder, attached with staples, and placed upright in the chromatography tank. Equilibration, when necessary, was allowed to occur overnight and development, following addition of the appropriate solvent system, was completed during the day, all procedures being carried out at room temperature (109).

4. Ion-Exchange Column Chromatography

Amberlite XE-97 and Dowex 50W-x4 resins were washed in water, screened by sedimentation and transferred to 12 cm x 0.6 cm columns. The resin was washed with 1 N HCl to get rid of fines and then with distilled water until the pH of the effluent was greater than 5, and finally it was washed with 0.1 M NaH_2PO_4 to a pH of 4. The sample was applied in a small volume and eluted with 0.1 M NaH_2PO_4 at pH 4.0. The effluent fractions were collected at 5 ml/hr in 1.2 ml fractions, and measured colorimetrically for esters by the modified Hestrin method (477,118).

5. Detection of Quaternary Ammonium Compounds

(i) Iodine Vapour

Individual spots or bands, corresponding to the R_f values of the quaternary ammonium compounds on thin-layer and paper chromatograms, were detected by placing them in a vessel containing iodine vapour (476). Spots of a brown to yellow colour appeared. As the spots exhibited a tendency to fade rapidly, they were outlined in pencil soon after removal from the vapour.

(ii) Free Choline

The measurement of free choline was according to Appleton (489,490,491). Choline chloride (C.P.) was dried over concentrated H_2SO_4 in vacuo, and a stable standard solution of 1 mg choline/ml water was prepared. Working standards containing 5 γ to 50 γ choline in 0.5 ml solution were prepared by suitable dilution in distilled water. A 0.5 ml sample (containing 0.01 to 0.5 μM choline) was treated with 0.2 ml of iodine reagent

(12.5 g KI and 9.8 g I_2 dissolved in distilled water to a total volume of 250 ml) and kept in an ice bath for 15 minutes. After centrifugation, the supernatant fluid was removed by aspiration, and the precipitate was dissolved in ethylenedichloride (reagent grade) to a volume of 10 ml. The optical density at 365 mμ was determined in a Beckman DB spectrophotometer. The molar extinction coefficient reported for choline was 2.7×10^4 cm^2/mole (490).

Part C. Isolation of Cholinester Hydrolases from Rat Brain

1. Homogenization

The procedure was essentially that of Parmar *et al.* (230). Adult black-hooded rats of either sex weighing 150 to 250 g were killed by decapitation, the brains removed, placed in a container surrounded by ice, and processed in the cold room. Tissues were homogenized in ice-cold 0.25 M sucrose (isotonic) using a glass hand homogenizer (Ace Glass Company, Vineland, New Jersey) with a clearance of 0.025 cm between pestle and cylinder. The final concentration of the homogenate was 10% w/v.

2. Centrifugation

The rat brain homogenate was fractionated by differential centrifugation using a modification of the techniques of Aldridge (228) and Brody and Bain (483). Nuclei and cell debris were obtained by centrifugation at 600 x g for 10 minutes and mitochondria at 10,000 x g for 15 minutes at 2°C in an International centrifuge, model HR 1, using the No.1608 cups. The microsomes were obtained by centrifugation at 30,000 x g for 2 hours at 0°C in the Spinco ultracentrifuge. Each fraction except the microsomes was washed and recentrifuged twice and then resuspended in a known volume of 0.25 M sucrose. The wash fluid from each fraction was added to the supernatant prior to the next centrifugation, and the supernatant from the preparation of the microsome fraction was the "supernatant" fraction. In later experiments, the nuclei were removed by centrifugation at 900 x g for 10 minutes and the mitochondria at 11,500 x g for 20 minutes (185).

3. Estimation of Protein Concentration

Protein concentration was routinely measured by the biuret method described by Gornall, Bardawill and David (484). The determination was based on the principle that proteins, containing peptide bonds, form a purple complex with copper salts in alkaline solution.

The biuret reagent (1.5 g $CuSO_4.5H_2O$, 6 g sodium potassium tartarate, and 300 ml 10% NaOH made up to 1 litre with distilled water) was a stable blue solution.

A standard curve was used for protein determinations. 0.1 to 0.6 ml of a standard albumin solution (100 mg bovine serum albumin/10 ml water) or an aliquot of unknown were made up to 1.5 ml volume with distilled water and 1.5 ml biuret reagent was added. The tubes were capped with Parafilm, mixed by inversion, and incubated at 38°C for 15 minutes. The optical density was determined at 540 mμ on a Junior Coleman spectrophotometer against a reagent blank of water and biuret reagent.

Part D. Preparation of Soluble Choline Dehydrogenase from Liver Mitochondria

The general method for determination of choline dehydrogenase activity spectrophotometrically was developed by Williams *et al.* (485,486).

1. Preparation of the Mitochondria (487)

Adult male black-hooded rats were decapitated, the livers were removed, chilled in ice, and homogenized in 4 volumes ice-cold 0.25 M isotonic sucrose. The nuclei and cell debris were removed by centrifugation at 600 x g for 10 minutes. The residue was twice washed with isotonic sucrose (3 ml/g original liver tissue), recentrifuged, and the supernatants added to that obtained during the first centrifugation. This mixed supernatant was centrifuged at 8,500 x g for 10 minutes. The supernatant was discarded and the residue was washed twice with isotonic saline (3 ml/g original liver), recentrifuged, and the supernatant discarded. The washed mitochondria were suspended with vigorous shaking in ice-cold distilled water (6.25 ml/g original liver). All centrifugations were carried out in an International HR-1 refrigerated centrifuge, at 2°C.

2. Purification of the Mitochondrial Enzyme

The water suspension of mitochondria was quick frozen in a mixture of dry ice and acetone and rapidly thawed at 37°C. The thawed mitochondria were then centrifuged at 25,000 x g for 30 minutes and the residue resuspended in ice-cold distilled water to the original volume (488). The mitochondria were homogenized with 20 volumes cold acetone, centrifuged, and the acetone treatment repeated. The residue was taken up in 20 volumes cold anhydrous diethyl ether and recentrifuged.

The mitochondria were partially air-dried on filter paper for 2 to 3 minutes, and the last traces of ether removed *in vacuo.* This powder was stable with respect to choline dehydrogenase activity for 2 weeks when stored at -5°C.

3. Preparation of Soluble Choline Dehydrogenase

In order to remove endogenous substrates, 20 mg dried mitochondria per ml 0.1 M sodium phosphate buffer (pH 6.8) were homogenized, centrifuged at 25,000 x g for 30 minutes and the residue then extracted with choleate.

A mixture of 20 mg mitochondrial residue and 6.0 mg sodium choleate (pH 6.8, added in a 3.0% concentrated solution) per ml of 0.1 M sodium phosphate buffer was homogenized in a glass hand homogenizer for 1 to 2 minutes and centrifuged for 30 minutes at 25,000 x g. The residue was resuspended in 1 ml buffer per 20 mg original mitochondrial preparation. Since acetone-dried mitochondria could be stored without loss of activity, a fresh supply of soluble enzyme was prepared as required.

4. Spectrophotometric Assay for Choline Dehydrogenase

The assays were carried out according to Williams (485,488). To a series of test tubes were added 1.0 ml Krebs-Ringer phosphate buffer at pH 6.8, 0.2 ml of 2.0% choline chloride (or water for controls), 0.5 ml of 10 mg % 2,6-dichlorophenolindophenol, and water to give a total volume of 2.2 ml. The contents of the test tube were transferred to matched cuvettes in a model DB Beckman spectrophotometer, and 1 ml of the mitochondrial suspension was directly pipetted into the mixture. A stop-watch was started after addition of half the enzyme, the cuvette quickly inverted, and replaced in the spectrophotometer. Optical density readings at 607 mμ were taken at 30 second intervals for 5 minutes.

Part E. Bioassay of Acetylcholine-Like Activity

1. The Eserine-Sensitized Frog Rectus Abdominis Method

The frogs (*Rana temporia*) were kept at 4°C for several days prior to use. The rectus abdominis muscle was excised and set up in a 3 ml organ bath at room temperature, and under tension of 5 g, approximately the resting tension on the muscle *in vivo*. The procedure was essentially that of MacIntosh and Perry (481) with modifications (473). The experiments were carried out in collaboration with A. Kato (473) of our laboratory.

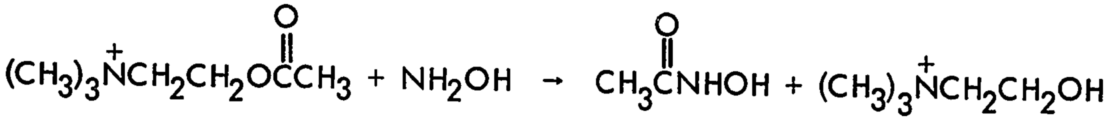
2. The Guinea Pig Ileum Method

Guinea pigs weighing 150 to 250 g were starved overnight. The terminal segment of the ileum was removed, divided into 10 cm sections, and washed clean with warm Tyrode's solution. A 4 cm segment was suspended in a 25 ml organ bath at 37°C under a tension of 0.5 g. The bath was aerated with a mixture of 95% O_2 and 5% CO_2. The procedure was that described by Kato (473) and was carried out in collaboration with A. Kato.

Part F. Determination of Cholinester Hydrolase Activity

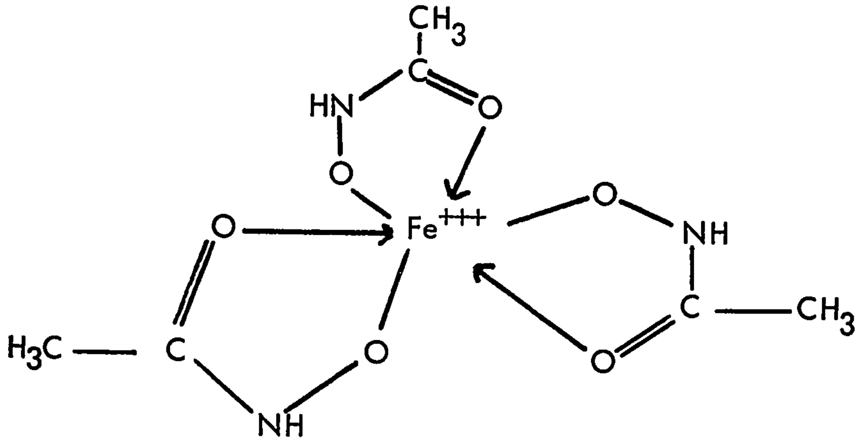
1. Detection of Esters by Hydroxamate Formation

The method was based on the rapid reaction with hydroxylamine which converts ACh stoichiometrically to acetyl-hydroxamate in alkaline solution (477) according to the equation:



$$(CH_3)_3\overset{+}{N}CH_2CH_2O\overset{\overset{O}{\|}}{C}CH_3 + NH_2OH \rightarrow CH_3\overset{\overset{O}{\|}}{C}NHOH + (CH_3)_3\overset{+}{N}CH_2CH_2OH$$

Ferric ions form coloured complexes with hydroxamates in acidic solutions with absorb maximally between 500 and 520 mµ (478).



Hestrin (477) developed an adaptation of this reaction for the quantitative determination of ACh and related choline esters in the concentration range of 1 to 5 mM. He found, however, that the method was not specific, giving reactions with simple esters, lactones and thioesters.

Friedman and Fraenkel (478) modified the Hestrin method for acetylcarnitine (AC) determination as follows: the AC was dissolved in 2.4 ml distilled water and the following reagents were added; 0.1 ml of a 28% $NH_2OH.HCl$ solution and 0.5 ml of a 14% NaOH solution, raising the pH of the solution to 12.6. The test tubes were then gently shaken and the solution acidified by the addition of 1.0 ml of 3N HCl followed by 1.0 ml of 5.0% $FeCl_3$ in 0.1N HCl to give a total volume of 5.0 ml. Each tube was mixed by inversion and the optical density was determined immediately on a Klett-Summerson colorimeter, using a No.54 filter. The concentration range for accurate measurement was between 0.02 and 5 µM of carnitine or choline ester per ml final solution. The optical density varied directly with the concentration of total ester bonds in solution.

2. Hydrolysis by the Cholinester Hydrolases

The action of the cholinester hydrolases on a variety of esters was measured according to Hestrin (477). The ester concentration remaining at the termination of the hydrolytic reaction was determined using the modified Hestrin method (478) described above.

3. Determination of the pH-Dependence of Enzymatic Activity

The determination of the pH-dependence of cholinester hydrolase activity was carried out according to Bergmann et al. (460), using the modified Hestrin ester determination (477,478).

For the pH range 5 to 8.5 the buffer used was 0.1M sodium phosphate, for higher pH values 0.1M borate buffer. The only cations present were Na^+ and K^+.

The concentrations of ester selected ensured that addition of the ester to the incubated mixture of enzyme and buffer at zero time did not dilute the mixture appreciably.

The rates were calculated by plotting ester hydrolysis as a function of time and extrapolating to zero time. The spontaneous non-enzymatic hydrolysis was measured at the same time and subtracted from the enzymatic rates.

4. Activity of Subcellular Fractions of Rat Brain

The cholinester hydrolase activity of the preparations of rat brain subcellular particles was determined according to Lawler (417) using the method described by Hestrin (477,478) following incubation in the presence of 3×10^{-3}M ACh Iodide, 2×10^{-2}M $MgCl_2$, 0.1 M NaCl, and 1×10^{-2}M potassium phosphate buffer (pH 7.0) at 37°C.

5. Determination of Cholinester Hydrolase Activities of Fresh Blood

For the fresh blood cholinester hydrolases, activity determinations were carried out according to Augustinsson (218,219) using the standard Hestrin detection of ester concentrations (477,478).

(i) Blood Sampling

Whole blood of human volunteers was taken up in heparinized tubes prepared by wetting the walls with a heparin solution (5-10 mg/ml) and subsequent drying in a gentle air stream to leave a coating of anticoagulant. The blood was centrifuged and the erythrocytes washed three times with 0.90% NaCl. The plasma and cells were then treated as follows:

(ii) Plasma Cholinesterase

5 μl of clear human plasma was expelled into 1.0 ml of 0.004M ester solution made up in M/15 phosphate buffer, pH 7.2; the pipette was rinsed three times with the reagent solution. The test tube was incubated exactly 30 minutes at 37°C after mixing. A standard of 1.0 ml buffer-ester solution without plasma was incubated. Addition of the hydroxylamine and NaOH solutions terminated the reaction and the amount of ester remaining was determined as described in Methods, Part F, Section 1.

5 μl of plasma was added to the standard immediately after addition of the hydroxylamine to allow for correction for non-enzymatic hydrolysis.

(iii) Erythrocyte Acetylcholinesterase

10 μl of washed erythrocytes was pipetted into 50 μl distilled water to hemolyze the cells, and the pipette rinsed three times in the solution. 10 μl of the hemolyzed cells was pipetted into 1.0 ml of

the buffer-ester solution as described above. The same procedure was followed as for the plasma enzyme described above.

6. Manometric Method of Determining Cholinester Hydrolase Activity

Cholinester hydrolase activity was measured by the Warburg manometric method described by Augustinsson (218,33). The method was based on the manometric estimation of the volume of carbon dioxide evolved from a bicarbonate-containing system by the acid formed in the ester hydrolysis.

Conical flasks, each of 15 ml volume, with one side bulb were employed. The flask constants were determined by the calibration method using mercury. The manometers were filled with Brodie's solution (23 g NaCl and 5 g sodium choleate in 500 ml water, coloured with methyl violet). The density of the solution was 1.034, 10,000 mm Brodie corresponding to 760 mm Hg.

The flasks and manometers were shaken at about 60 complete oscillations per minute. In most cases, the temperature of the water thermostat was 37 ± 0.05°C.

A. Measurement of Activity

The volume of the reaction mixture was 2.0 ml. Into the main compartment of the flask 1.6 ml enzyme solution was pipetted and into the side bulb 0.4 ml substrate solution. Substrate and enzyme preparations were dissolved in a bicarbonate-buffer solution (bicarbonate-Ringer's solution containing 100 ml 0.90% NaCl, 30 ml 1.26% $NaHCO_3$, 2 ml 1.20% KCl and 2 ml 1.76% $CaCl_2 : 6\ H_2O$).

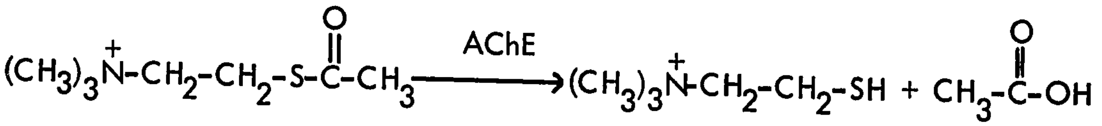
The hydrolysis was carried out in a gas mixture of 95% O_2 and 5% CO_2 by volume. The solutions were saturated with the gas mixture and the flasks filled after attachment to the manometers. Before the enzyme solutions were mixed with the contents of the side bulb, temperature equilibrium was attained by shaking in the water bath for 15 minutes.

The contents of the flasks (8 per experiment) were mixed at one minute intervals and each manometer was read at 6 to 10 minute intervals, one minute between each manometer reading. Readings were continued for 40 minutes. In all experiments a thermobarometer was used, filled with the same volume of the substrate solution of the same concentration as that in the reaction mixtures. Corrections for thermobarometer changes, due to alterations in temperature and pressure, were made.

The results were recorded in tabular form and the amount of CO_2 released expressed in µl was plotted against time. The initial slope of the curve, minus the slope of the curve for non-enzymatic hydrolysis, was an expression of enzymatic activity.

Part G. Determination of Cholinester Hydrolase Inhibition

A modification of the method of McOsker and Daniel (479) was used to determine the inhibition of AChE. Acetylthiocholine (AcTCh) was utilized as substrate and the determination of free sulphydryl groups following hydrolysis by reaction with sodium nitroprusside was a direct assay of enzymatic activity.



$$(CH_3)_3\overset{+}{N}-CH_2-CH_2-S-\overset{\overset{O}{\|}}{C}-CH_3 \xrightarrow{AChE} (CH_3)_3\overset{+}{N}-CH_2-CH_2-SH + CH_3-\overset{\overset{O}{\|}}{C}-OH$$

Several concentrations of substrate and inhibitor were incubated with purified erythrocyte AChE (Sigma). The incubation mixture consisted of 0.20 ml AcTCh Iodide, 0.50 ml Tris buffer (0.125M, pH 7.4), 0.20 ml 3.5M NaCl, 0.1 ml inhibitor, and distilled water to give a total volume of 1.30 ml.

The incubation mixture was equilibrated to 37°C, and the reaction was begun by addition of 0.10 ml of a 0.1% enzyme solution.

The reaction was terminated at varying intervals by addition of 0.10 ml of 25% trichloroacetic acid (w/v). Aliquots of the reaction mixture were withdrawn and the sulphydryl concentration was determined using a modification of the method described by Grunert and Phillips (480).

Into a 3 ml Beckman cuvette the following reagents were pipetted: 2.0 ml saturated NaCl solution, 0.40 ml Na_2CO_3-NaCN solution (21.2 g anhydrous Na_2CO_3 and 0.44 NaCN per 100 ml water), and 0.40 ml Na nitroprusside solution (27 mg/ml). The reagents were mixed and a 0.2 ml aliquot of the sample was immediately added. The optical density at 520 mμ was read in a model DB Beckman spectrophotometer exactly 30 seconds after addition of the sample. Appropriate blanks containing heat-inactivated enzyme and substrate or omitting the substrate were used.

MATERIALS

Acetylcarnitine hydrochloride, carnitylcholine dichloride and acetylcarnitylcholine dichloride were synthesized in our laboratory by G. Kato (Methods, Part A).

Partially purified bovine erythrocyte acetylcholinesterase (AChE) and horse serum cholinesterases (ChE) were obtained from Sigma Chemical Co., St. Louis, Mo. Human serum ChE was supplied by Schwarz Bioresearch, Inc., Orangeburg, New York.

Succinyldicholine chloride was obtained from Glaxo-Allenbury's (Canada) Ltd., Montreal, Quebec.

RESULTS

Section A. Preliminary Experiments

1. Reproducibility of Colorimetric Methods

(i) Modified Hestrin Method

Since in most of the experiments described, the velocity of reaction was measured colorimetrically, it was necessary to establish the reliability, sensitivity and reproducibility of the standard methods used. The Hestrin method (477), modified by Friedman and Fraenkel (118), was used to measure quantitatively the hydrolysis of several esters. The extent, therefore, of the reaction between alkaline hydroxylamine and the pure esters was determined. For all esters, the optical density in Klett units increased linearly with increasing concentration of the pure ester in solution (Figure 1, B). The readings per unit concentration recorded on a Klett-Summerson photoelectric colorimeter (e.g., 1 µMole ACh iodide gave a reading of 92 Klett units) varied for some of the esters. The standard curves were repeated regularly when fresh reagents were made up or new preparations of synthetic esters were synthesized. The following values represent the number of Klett units per µMole ester: acetylcholine, 92; acetyl-ß-methylcholine, 91; butyrylcholine,92; succinyldicholine chloride, 117; dl-acetylcarnitine, 74; dl-carnitylcholine, 86; and dl-acetylcarnitylcholine, 172.

Figure 1

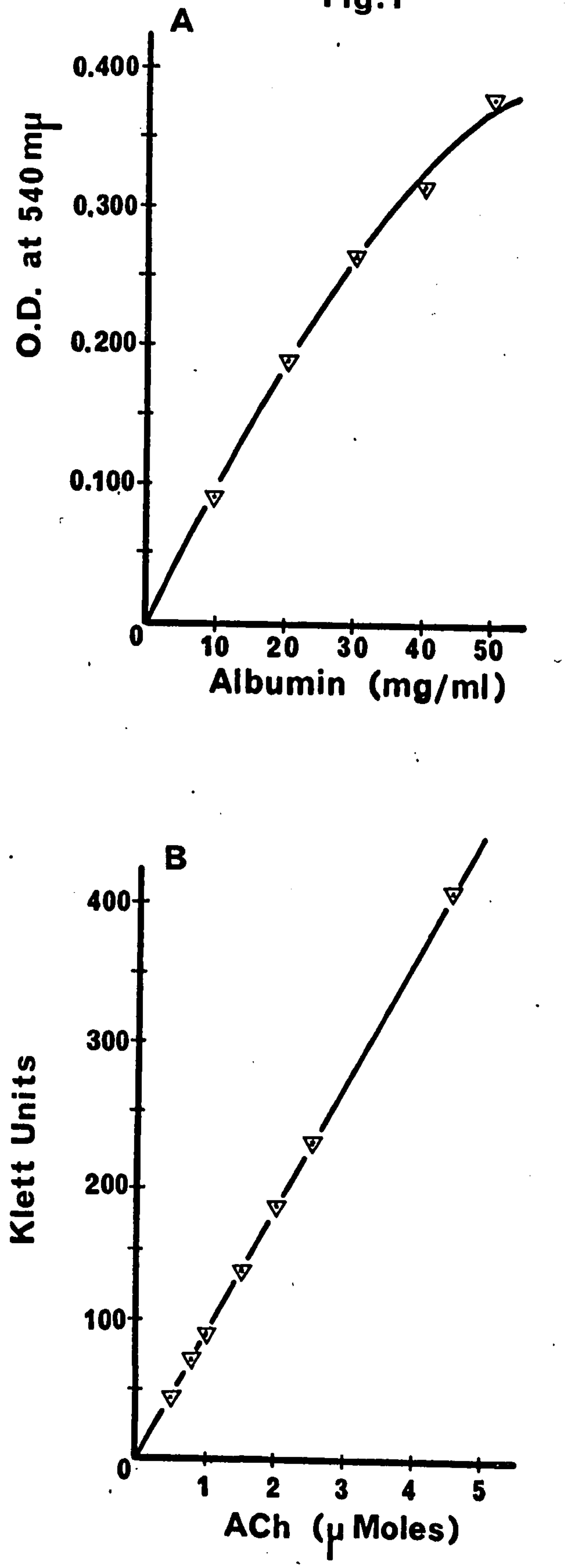
A. Standard Biuret Curve of Protein Concentration

Protein concentrations of a standard bovine serum albumin solution in mg per ml in a total volume of 1.5 ml read at 540 mµ in a Junior Coleman colorimeter. (TABLE I).

B. Standard Hestrin Curve of Acetylcholine Iodide Concentration

The ester concentration in µMoles ACh was read in a Klett-Summerson photoelectric colorimeter with a green No.54 filter. The curve was calculated according to the method of least squares from the results of 10 experiments.

Fig.1


A
O.D. at 540 mμ
0.400
0.300
0.200
0.100
0
10
20
30
40
50
Albumin (mg/ml)
B
Klett Units
400
300
200
100
0
1
2
3
4
5
ACh (μ Moles)

(ii) Biuret Method

The biuret method (484) was used routinely for the measurement of protein concentrations. The standard protein employed was bovine serum albumin and it was found that increasing concentrations of protein increased the optical density linearly to a concentration of 30 mg albumin per ml. The slopes obtained for all the standard curves determined corresponded to one another (Figure 1,A). (TABLE I). Throughout this work, data are recorded as follows: in the text, Tables 1-11 and Figures 1-37 are presented. Data from which Figures 1-37 originated are recorded in the Appendix, TABLES I-XLI, at the end of this thesis.

(iii) Free Choline Method

The "free choline" method of Appleton (489) was used to measure concentrations of several quaternary nitrogen compounds. For each compound tested, a linear standard curve was obtained the slope of which varied according to the compound (Figure 2).

(iv) Nitroprusside Determination

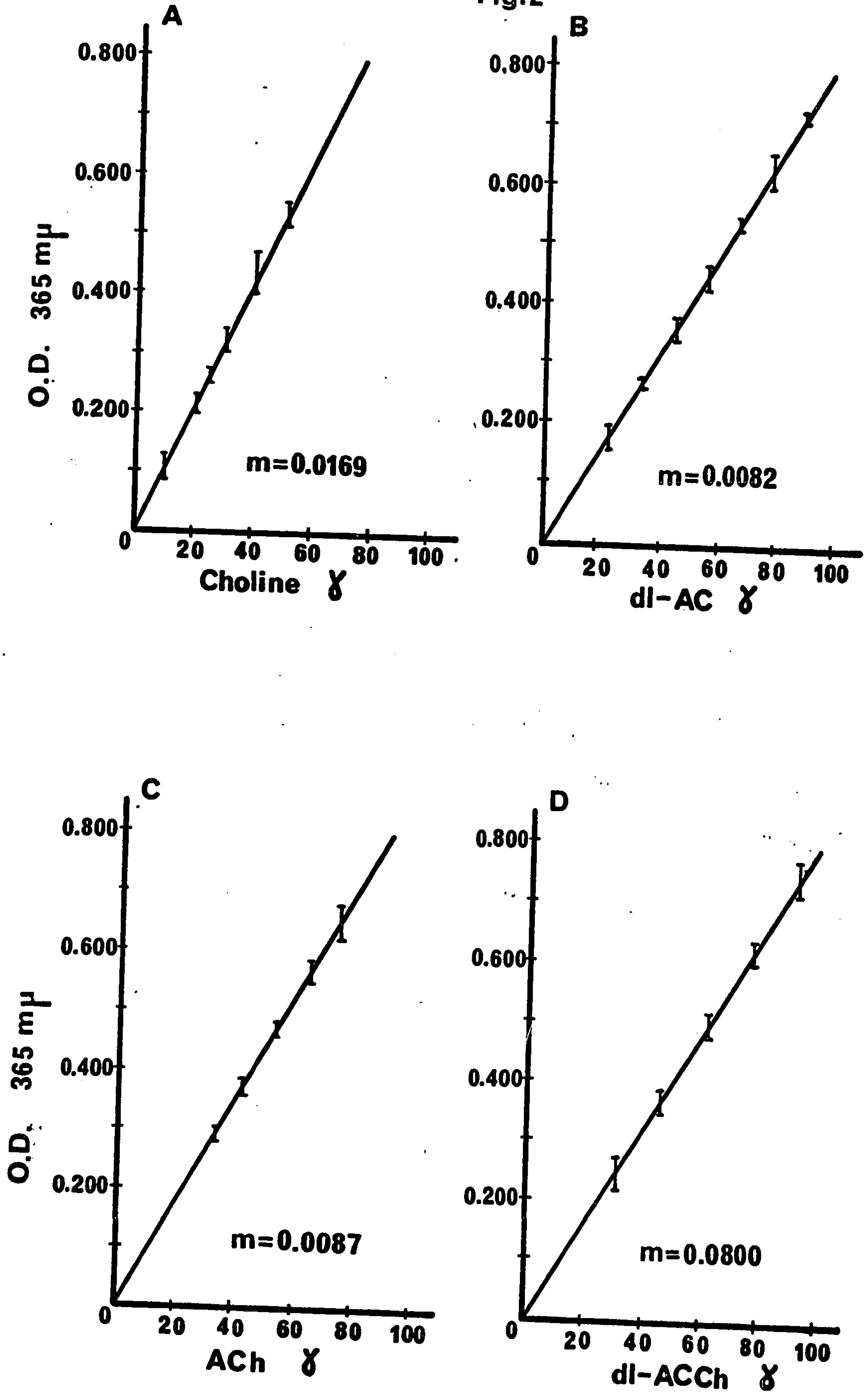
The nitroprusside method of Grunert and Phillips (480) was used to determine the concentration of sulphydryl groups released by the enzymatic hydrolysis of acetylthiocholine. Consistency of the timing of the colour reaction at 30 seconds was found to be essential for reproducibility.

Figure 2

Iodine Standard Curves for Quaternary Compounds

Standard curves obtained employing the "free choline" method for choline chloride (A), dl-acetylcarnitine hydrochloride (B), acetylcholine iodide (C), and dl-acetylcarnitylcholine chloride (D). Concentrations are presented in μg or γ, and the colour at 365 mμ in O.D. units. Vertical bars represent the standard errors from the mean.

Fig.2


A
B
C
D
O.D. 365 mμ
0.800
0.600
0.400
0.200
0
20
40
60
80
100
m=0.0169
Choline γ
m=0.0082
dl-AC γ
m=0.0087
ACh γ
m=0.0800
dl-ACCh γ

2. The Purity of Various Preparations of Acetylcarnitine and Other Carnitine Esters

With preparations of carnitine esters prepared in our laboratory by G. Kato, a new standard curve was prepared using the modified Hestrin method (118) in order to ensure the purity of the compound. The preparations were also run in a thin-layer chromatographic system to ensure their purity.

3. Stability of Choline and Carnitine Esters at Alkaline pH

Determination of ester concentration was carried out using the Hestrin method modified by Friedman and Fraenkel (118). One μMole acetylcholine (ACh), acetylcarnitine (AC), carnitylcholine (CCh), and acetylcarnitylcholine (ACCh) were incubated at room temperature in 1.4 ml volumes of buffers of the given pH's. It was found that incubation of the monoquaternary esters ACh and AC in the pH range 7.0 to 9.0 caused no hydrolysis in 20 minutes, whereas when the pH was raised to 12.0 increasing hydrolysis occurred (recorded as decreases in Klett units). With the bisquaternary esters, however, alkaline hydrolysis was more pronounced,being detectable even at pH 7.5 to 8.0 (Figure 3).

4. Methods of Terminating Enzymatic Activity

Various methods of terminating the activities of different enzyme

Figure 3

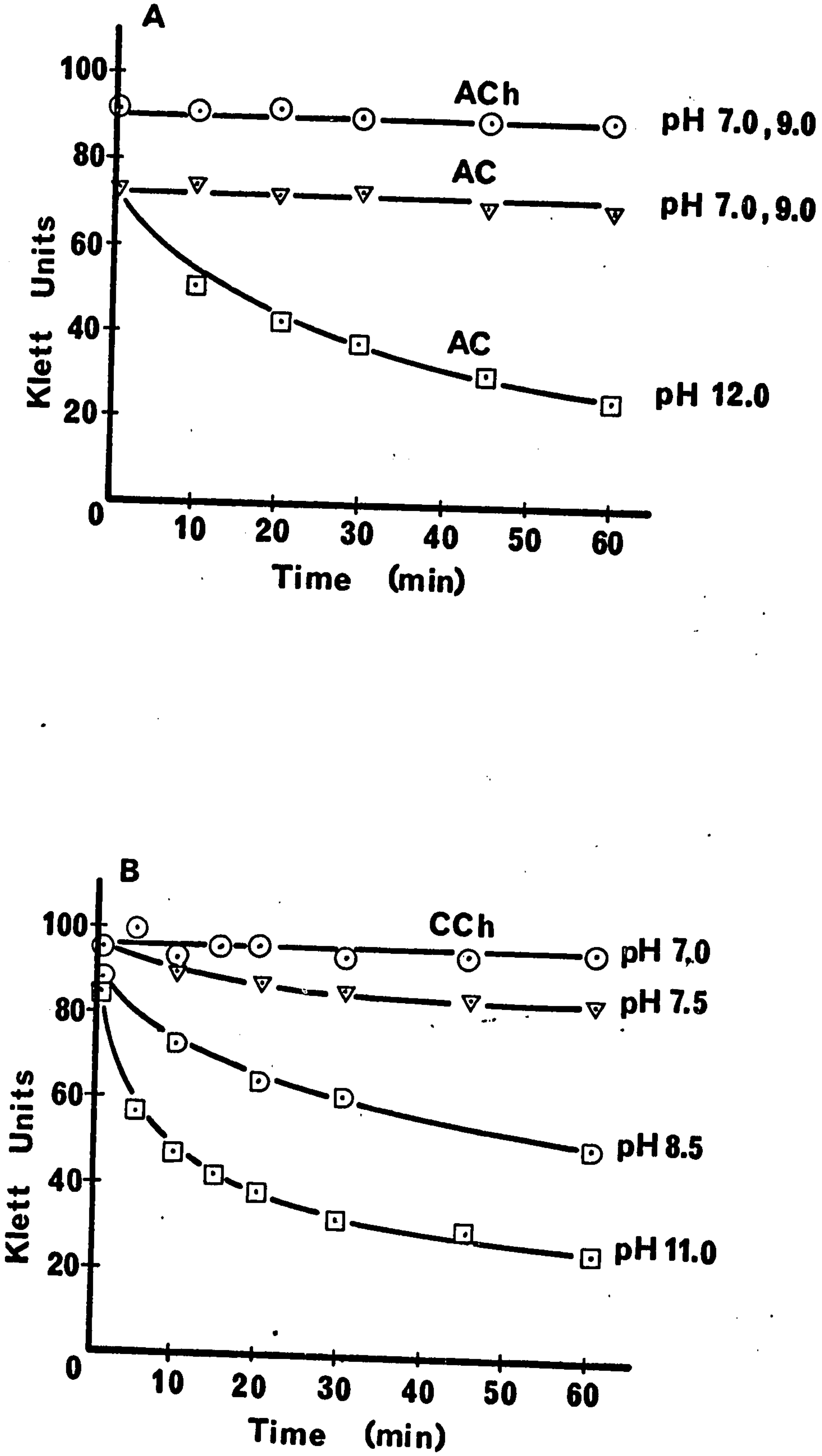
A. Alkaline Hydrolysis of Acetylcholine Iodide and dl-Acetyl-Carnitine Hydrochloride

One µMole of the pure compounds was incubated at room temperature for the indicated time in a volume of 1.4 ml 0.1M sodium phosphate buffer (pH 7.0 - 8.5), 0.1M sodium borate buffer (pH 8.5 - 10.5), or 0.1M sodium carbonate-sodium bicarbonate solution (pH 11.0). The amount of ester remaining in the mixtures was determined by the modified Hestrin method (118) and the values presented in Klett units. (TABLE II).

B. Alkaline Hydrolysis of dl-Carnitylcholine Chloride

The same procedures were carried out as described above.

Fig. 3


A
100
80
60
40
20
0
Klett Units
10
20
30
40
50
60
Time (min)
ACh
pH 7.0, 9.0
AC
pH 7.0, 9.0
AC
pH 12.0
B
100
80
60
40
20
0
Klett Units
10
20
30
40
50
60
Time (min)
CCh
pH 7.0
pH 7.5
pH 8.5
pH 11.0

preparations were investigated.

(i) Trichloroacetic Acid

Trichloroacetic acid solutions (TCA) were added to incubation mixtures in order to lower the pH of the solution and precipitate enzyme protein. The pH could not be decreased below 3, as lower pH's caused acid-catalyzed hydrolysis of both choline and carnitine esters (495). The esters were incubated with enzyme in the presence of 0.02M $MgCl_2$, 0.10M NaCl, and 0.01M potassium phosphate buffer (pH 7.0) according to Lawler (417). It was found that for such a mixture in the presence of 1 mg enzyme protein in a total volume of 1.0 ml, the addition of 0.1 ml 3% TCA (w/v) gave a pH of 3.10 ± 0.05. This was sufficient to terminate enzymatic activity with the subcellular fractions of rat brain.

(ii) Heat Inactivation

The possibility of terminating enzymatic activity by placing the incubation mixtures in a boiling water bath for 1 to 10 minute intervals was investigated with the subcellular fractions of rat brain. This produced flocculant precipitates and turbid solutions which were not suitable for colorimetric assays. This method was abandoned for crude enzyme preparations.

(iii) Alkaline Hydroxylamine Reagents

For the purified enzyme preparations (Sigma), it was found that the addition of the alkaline hydroxylamine colour reagents employed in the modified Hestrin colorimetric method (118) terminated the enzymatic activity immediately and that the small amount of protein in the incubation mixtures did not interfere with the colorimetric assays.

Section B. Manometric Assay of Cholinester Hydrolase Activity

The choice of assay methods for the cholinester hydrolases is dependent mainly on the purpose of the investigation, available equipment, and number of assays to be performed. One of the most commonly employed methods is the Warburg manometric technique, which can be applied in almost any investigation with any type of enzyme preparation. The disadvantage of the technique is that the pH of the incubation medium cannot be altered.

1. Hydrolysis of Acetylcholine Iodide by Acetylcholinesterase

The manometric method was investigated for possible use in following the hydrolysis of dl-acetylcarnitylcholine chloride before the nature of the products was examined.

Experiments were carried out exactly as given in Methods, Part F, 6. To determine the reproducibility of the method, the hydrolysis of acetylcholine iodide (ACh) by pure bovine erythrocyte acetylcholinesterase (AChE) (Sigma) was studied to check the mercury calibration of the manometers. The main compartment of the flasks contained 1.6 ml enzyme solution (2 mg AChE/15 ml bicarbonate-Ringer's solution) and the side arm 0.010 g ACh in 0.4 ml bicarbonate-Ringer's solution. Figure 4 shows the excellent reproducibility of this enzymatic assay. The variation between manometric flasks in these experiments agreed very closely with the differences determined for calibration by the mercury method.

The effect of varying the enzyme concentration (AChE) upon the hydrolysis of ACh was studied manometrically. The results in Figure 5 show the relationship to be linear. The reproducibility was excellent, the maximum deviation from the mean for any value being 7 µl CO_2.

The effect of varying ACh concentration upon hydrolysis by AChE was also investigated. From Figure 5 it could be seen that marked substrate inhibition took place above a concentration of 5 mg ACh iodide in the presence of 0.2 mg purified AChE (Sigma).

In all experiments, controls for non-enzymatic hydrolysis consisted of incubation in the presence of heat-inactivated enzyme which had been placed in a boiling water bath for 10 minutes. Values for non-enzymatic hydrolysis were subtracted from the values obtained with the active enzyme.

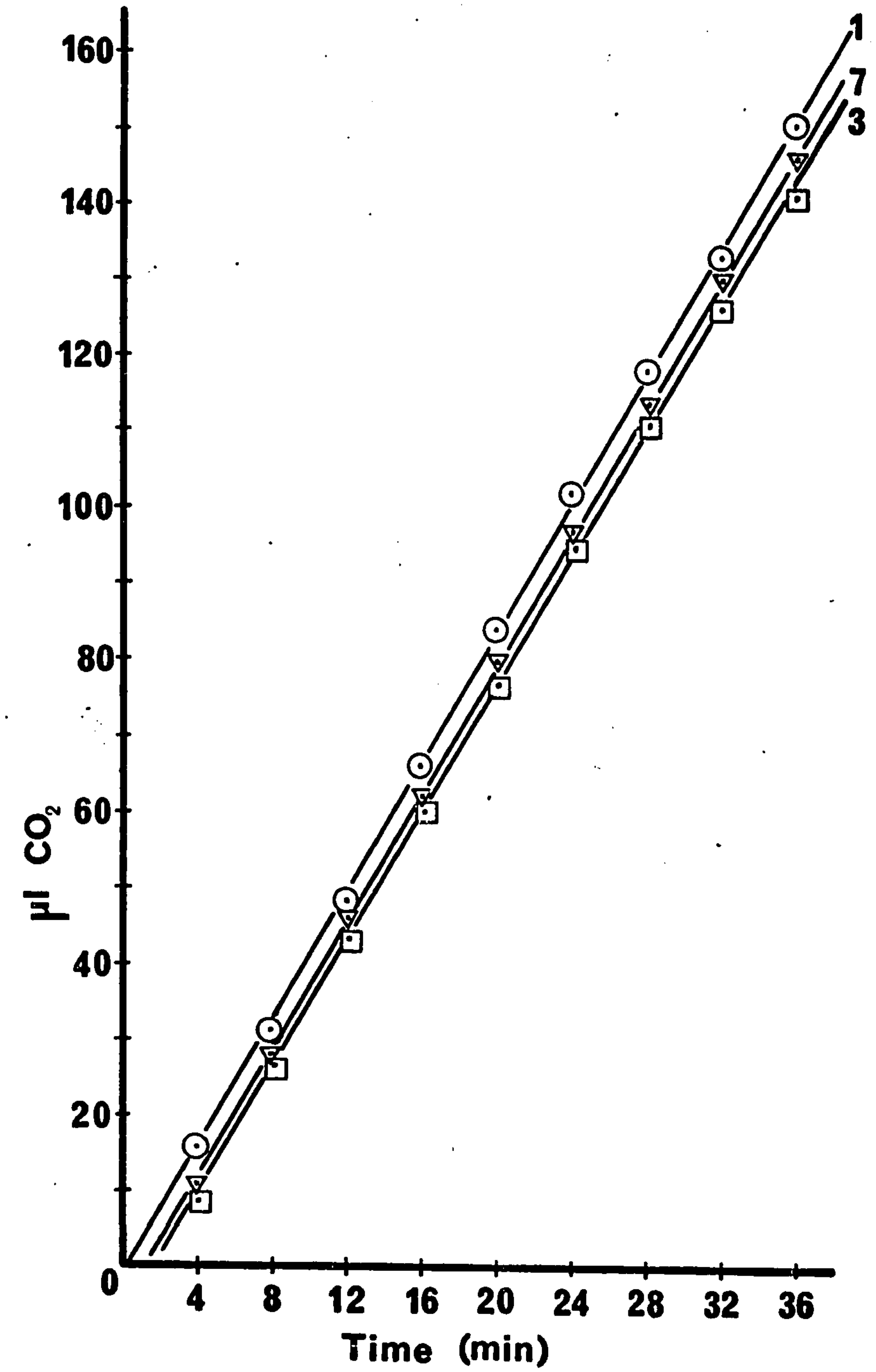
2. Hydrolysis of dl-Acetylcarnitylcholine Chloride by Acetylcholinesterase (Sigma)

Figure 4

Reproducibility of the Manometric Determination of Acetylcholinesterase Hydrolysis of Acetylcholine Iodide

The standard manometric method was employed (Methods, Part F,6). Incubation took place in the presence of 0.010 g ACh iodide in 0.4 ml bicarbonate-Ringer's solution and 1.6 ml enzyme solution (2 mg AChE/15 ml bicarbonate-Ringer's solution), (pH 7.4) at 37°C for 40 minutes. Only 3 of the 7 experimental flasks are presented graphically. The maximum difference existed between flasks 1 and 3, the values for all the 5 other flasks lying between these extremes. The variation in values was accounted for by the different flask constants.

Fig. 4



μl CO2
160
140
120
100
80
60
40
20
0
4
8
12
16
20
24
28
32
36
Time (min)
1
7
3

Figure 5

Effects of Varying Enzyme and Substrate Concentrations on Hydrolysis of Acetylcholine Iodide by Acetylcholinesterase

A. Enzyme Concentration

The standard manometric method was employed. Incubation took place in the presence of 0.010 g ACh Iodide and varying concentrations of AChE (4 mg/15 ml bicarbonate-Ringer's solution) at 37°C for 40 minutes. The values presented are the initial velocities in µl CO_2 released per 30 minutes, the mean values of 4 determinations. (TABLE III).

B. Substrate Concentration

The standard manometric method was employed. Incubation took place in the presence of 0.2 mg AChE (Sigma) and varying concentrations of ACh Iodide at 37°C for 40 minutes. The values presented are the mean values of 5 determinations of initial velocities in µl CO_2 released per 30 minutes. (TABLE IV).

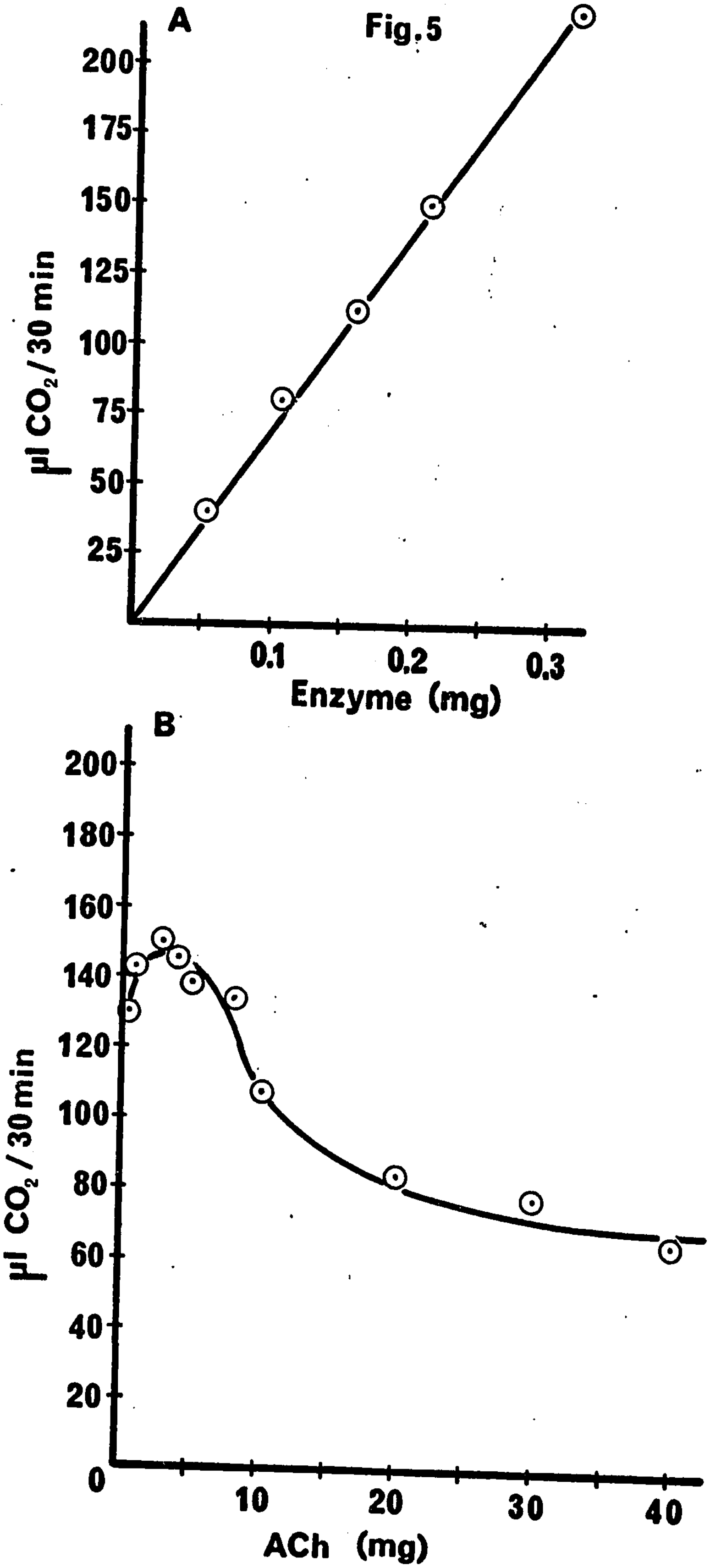


Fig.5

A
μl CO$_2$/30 min
200
175
150
125
100
75
50
25
0.1
0.2
0.3
Enzyme (mg)
B
μl CO$_2$/30 min
200
180
160
140
120
100
80
60
40
20
0
10
20
30
40
ACh (mg)

The manometric method, the reliability of which had been established with ACh as substrate, was studied for enzymatic dl-acetylcarnitylcholine (dl-ACCh) hydrolysis.

The non-enzymatic hydrolysis of the compound was marked in this system. The levels were very much higher than for ACh as is seen in Figure 6,A. This was due to the lack of stability of bisquaternary compounds at pH 7.4, the pH of the buffer system. When the readings for non-enzymatic hydrolysis were subtracted from those obtained in the presence of active AChE (Figure 6,B), the readings were very low compared to those for ACh. A possible explanation of the low amounts of carbon dioxide released from the buffer might be the later finding that the product of hydrolysis is the weak acid acetylcarnitine, rather than acetic acid.

Because such high concentrations of substrate (2.5 to 7.5 mg dl-ACCh) were required for this method, and in view of the very low activities recorded, the method was of little use in the general investigations. This was true especially in the light of the very limited quantities of dl-ACCh available for the entire investigation.

Section C. Hydrolysis of Choline and Carnitine Esters by the Cholinester Hydrolases of Rat Brain

A study was made of the activity of the cholinester hydrolases of fresh rat brain subcellular fractions on acetylcholine iodide (ACh), dl-

Figure 6

A. Non-Enzymatic Hydrolysis of dl-Acetylcarnitylcholine Chloride

The standard manometric method was employed (Methods, Part F,6). Incubation was in the presence of 0.2 mg heat-inactivated bovine erythrocyte AChE (Sigma), placed in a boiling water bath for 10 minutes, and varying concentrations of dl-ACCh, at 37°C for 40 minutes. The values presented represent the µl CO_2 released with time.

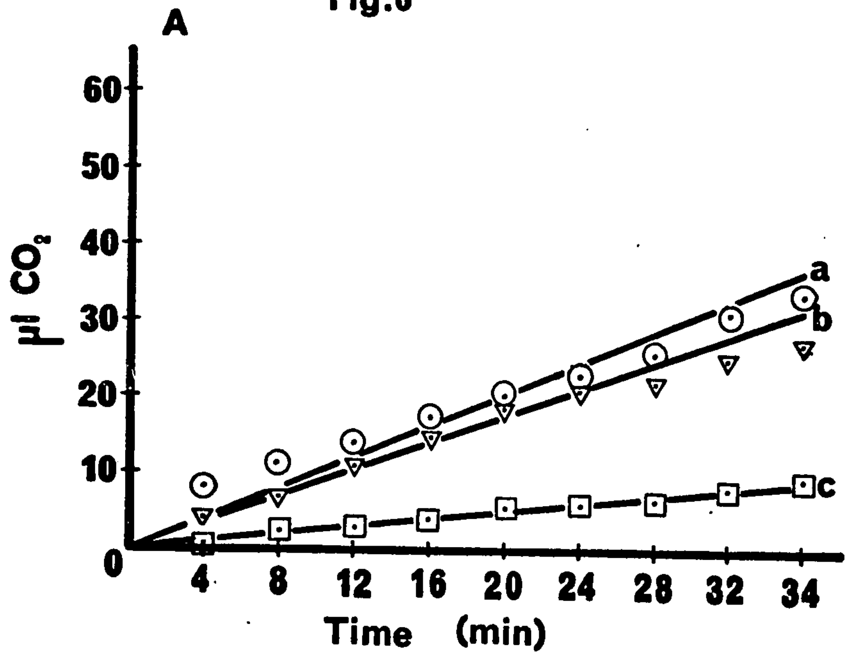
(a) 7.5 mg dl-ACCh
(b) 5.0 mg dl-ACCh
(c) 2.5 mg dl-ACCh

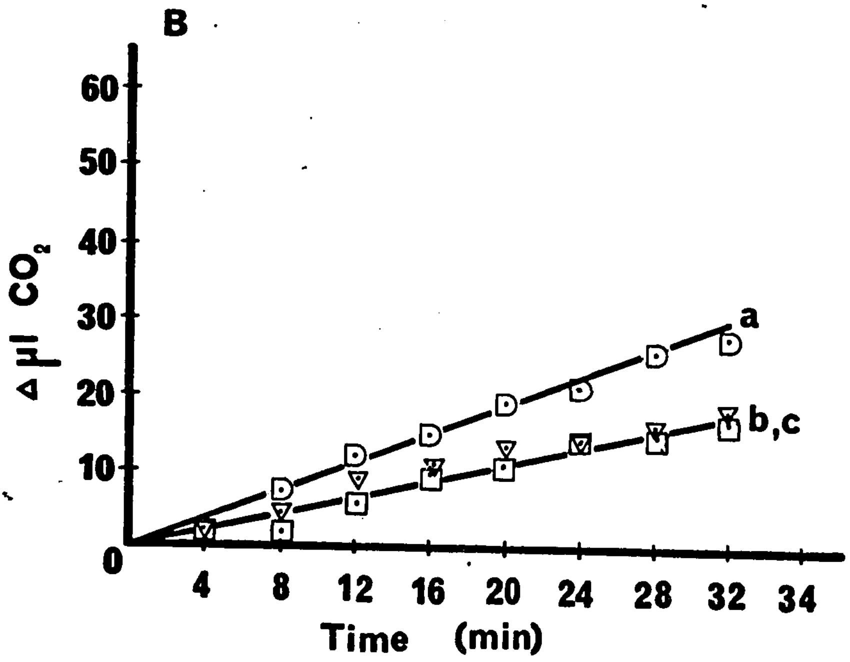
B. Enzymatic Hydrolysis of dl-Acetylcarnitylcholine Chloride by Acetylcholinesterase

The standard manometric method was employed. Incubation took place as above in the presence of 0.2 mg active AChE. The values presented are the µl CO_2 released during enzymatic activity minus µl CO_2 released non-enzymatically with time for varying substrate concentrations.

(a) 7.5 mg dl-ACCh
(b) 5.0 mg dl-ACCh
(c) 2.5 mg dl-ACCh

Fig.6


A
µl CO$_2$
60
50
40
30
20
10
0
a
b
c
4
8
12
16
20
24
28
32
34
Time (min)


B
Δ µl CO$_2$
60
50
40
30
20
10
0
a
b,c
4
8
12
16
20
24
28
32
34
Time (min)

acetylcarnitylcholine chloride (dl-ACCh), dl-carnitylcholine chloride (dl-CCh) and dl-acetylcarnitine (dl-AC).

The rat brain subcellular fractions were isolated as described in Methods, Part C. The total cholinester hydrolase activity of the preparations was determined by the modified Hestrin method (118) after incubation at 37°C in the presence of the indicated concentrations of substrate (1 to $4.5 \times 10^{-3}M$), $0.02M\ MgCl_2$, $0.10M\ NaCl$, and $0.01M$ potassium phosphate buffer (pH 7.0) in a total volume of 1.0 ml as described by Lawler (417).

The concentration of protein was measured by the biuret method (Methods, Part C, 3) and the activity was expressed in µMoles of substrate hydrolyzed per mg protein per 30 minutes.

The reaction was terminated by the addition of 0.1 ml 3% TCA (w/v).

The results of these experiments are presented in Figures 7 and 8. No hydrolysis by any subcellular fraction of rat brain of dl-CCh or dl-AC was found. The hydrolysis, however, of both ACh and dl-ACCh were completely abolished by $3 \times 10^{-5}M$ eserine sulphate indicating that the enzyme or enzymes involved were the cholinester hydrolases. Hydrolysis of dl-ACCh and ACh occurred in all fractions, being most marked in the combined microsomal-supernatant fraction. That all sub-fractions hydrolyzed both substrates reflected the lack of well-defined localization of the two cholinester hydrolases in any one fraction (228,229). Having established, therefore, the hydrolysis of dl-ACCh by rat brain enzymes, purified enzymes (Sigma) were investigated

Figure 7

Hydrolysis of Acetylcholine Iodide and dl-Acetylcarnitylcholine Chloride by Fresh Rat Brain Subcellular Fractions

A. Nuclear Fraction

0.1 ml freshly prepared nuclear fraction of a whole rat brain homogenate in 0.25M sucrose (900 x g for 15 min) and varying concentrations of the substrates, ACh and dl-ACCh, were incubated at 37°C for 30 minutes in a total volume of 1.0 ml in a mixture containing 0.02M $MgCl_2$, 0.10M NaCl, and 0.01M potassium phosphate buffer, pH 7.2. The total cholinester hydrolase activity was determined by the modified Hestrin method (118) and the activity was expressed in μMoles substrate hydrolyzed per mg protein per 30 minutes. Eserine sulphate (3×10^{-5}M) abolished enzymatic activity. Controls for non-enzymatic hydrolysis consisted of incubation with enzyme which had been heat-inactivated at 80°C for 60 minutes and were subtracted from the enzymatic hydrolysis determinations.(TABLES V and VI).

(a) ACh iodide
(b) dl-ACCh chloride
(c) Both substrates plus 3×10^{-5}M eserine sulphate

B. Mitochondrial Fraction

The same procedures described above were followed, employing 0.1 ml mitochondrial fraction (10,000 x g for 20 minutes).(TABLES VII and VIII).

(a) ACh iodide
(b) dl-ACCh chloride
(c) Both substrates plus 3×10^{-5}M eserine sulphate

Fig.7

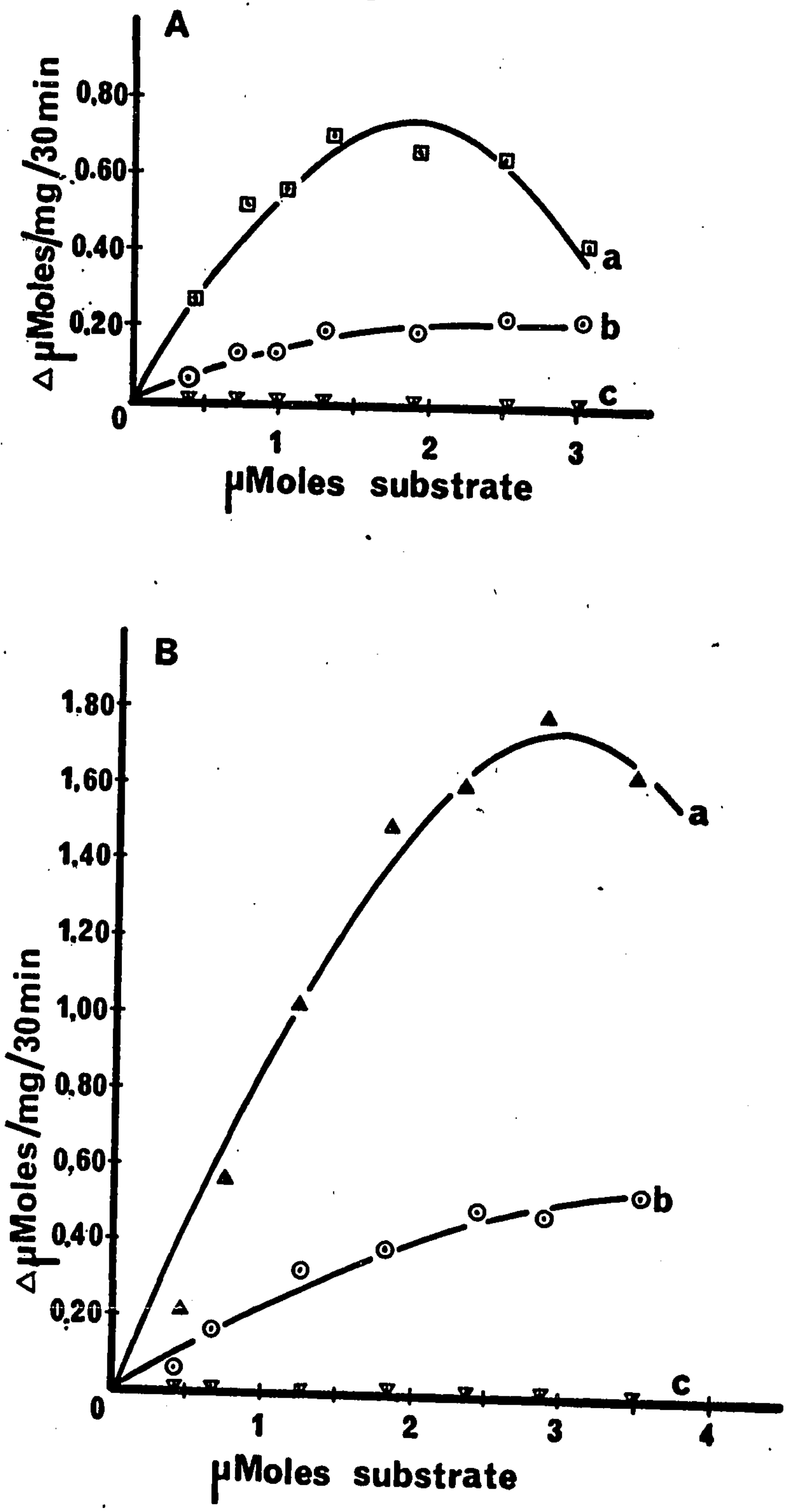

A
0,80
0,60
0,40
0,20
0
Δ μMoles/mg/30 min
a
b
c
1
2
3
μMoles substrate
B
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0
Δ μMoles/mg/30 min
a
b
c
1
2
3
4
μMoles substrate

Figure 8

Hydrolysis of Acetylcholine Iodide and dl-Acetylcarnitylcholine Chloride by Fresh Rat Brain Subcellular Fractions

C. Combined Microsomal and Supernatant Fractions

The same procedures were employed as described for Figure 7, employing 0.1 ml of the combined microsomal and supernatant fractions. (TABLES IX and X).

(a) ACh iodide
(b) dl-ACCh chloride
(c) Both substrates plus 3×10^{-5}M eserine sulphate

Figure 8

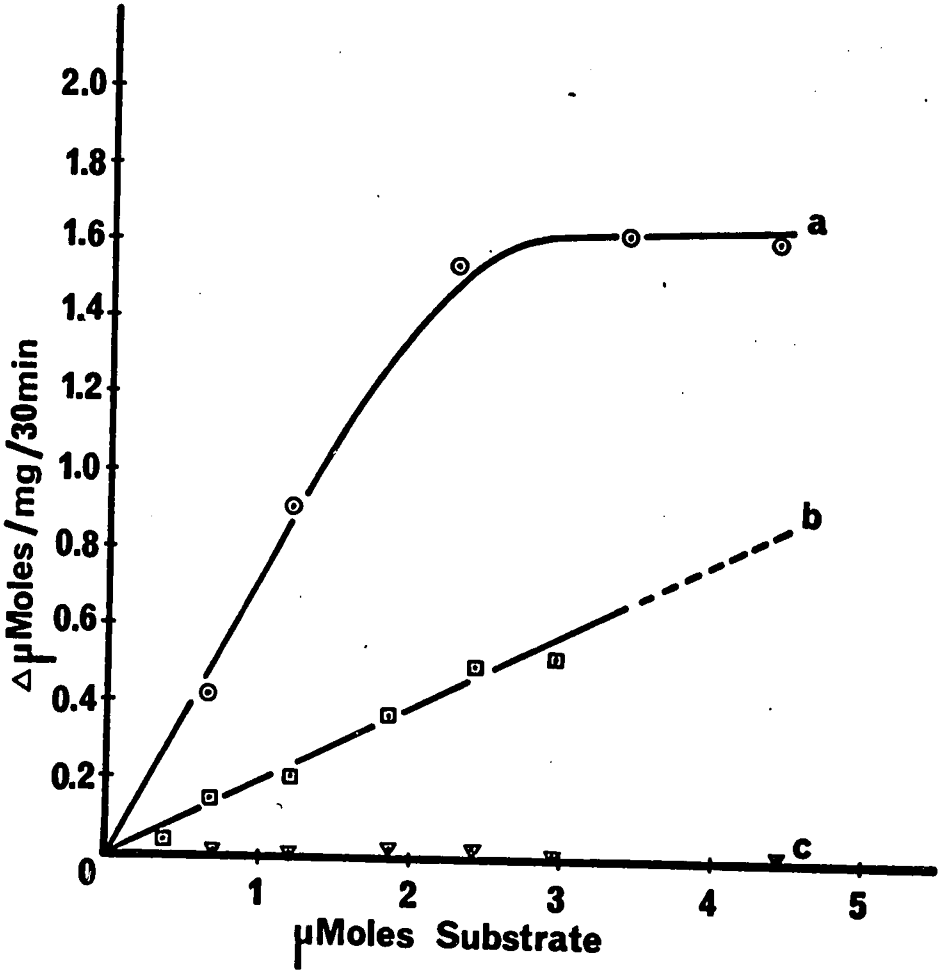
Hydrolysis of Acetylcholine Iodide and dl-Acetylcarnitylcholine Chloride by Fresh Rat Brain Subcellular Fractions

C. Combined Microsomal and Supernatant Fractions

The same procedures were employed as described for Figure 7, employing 0.1 ml of the combined microsomal and supernatant fractions. (TABLES IX and X).

(a) ACh iodide
(b) dl-ACCh chloride
(c) Both substrates plus 3×10^{-5}M eserine sulphate

Fig. 8



2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Δ μMoles/mg/30min
a
b
c
1
2
3
4
5
μMoles Substrate

for cholinester hydrolase activity toward this compound.

Section D. Hydrolysis of Choline and Carnitine Esters by Cholinester Hydrolases of Fresh Human Blood

The hydrolysis of several esters by the cholinester hydrolases of freshly drawn human blood was examined. The blood was drawn and the crude enzymes prepared as in Methods, Part F,5. Varying concentrations of acetylcholine iodide (ACh), dl-acetylcarnitylcholine chloride (dl-ACCh), l-carnitylcholine chloride (l-CCh), and dl-acetylcarnitine (dl-AC) were incubated with erythrocyte and plasma enzymes at 37°C for the times specified in Tables 1 and 2, and in Figure 9. The enzymatic reactions were terminated by the addition of the alkaline hydroxylamine colour reagents and the amount of ester hydrolyzed was determined by the modified Hestrin method (118). Non-enzymatic hydrolysis controls consisted of incubation in the presence of heat-inactivated enzyme which had been placed in a boiling water bath for 20 minutes.

From Tables 1 and 2, it could be seen that the activity measured was that of the cholinester hydrolases as both enzymes were completely inhibited by 10^{-5}M eserine sulphate.

From Table 1, in which the hydrolysis by 50 µl washed erythrocytes was measured, 2 µMoles ACh were completely hydrolyzed in 10 minutes. For l-CCh and dl-AC, however, no hydrolysis occurred in 30 minutes. The

Table 1

Hydrolysis of Choline and Carnitine Esters

(Fresh Human Erythrocytes)

Time	Active Enzyme 50 µl	Inact. Enzyme 50 µl	ACh 2 µM	dl-ACCh 2 µM	l-CCh 2 µM	dl-AC 2 µM
min			K.U.	K.U.	K.U.	K.U.
0	+	-	185±2.0	345±3.0	172±1.2	148±2.1
0	-	+	185±2.1	345±3.0	173±1.4	148±0.9
5	+	-	24±1.0	305±3.1	172±1.0	147±1.5
5	-	+	184±2.1	343±2.7	172±1.5	148±0.5
10	+	-	0±1.0	270±2.5	172±2.0	148±1.0
10	-	+	185±2.2	342±2.8	171±0.9	147±1.1
20	+	-	0±0.5	257±2.3	173±2.1	149±0.9
20	-	+	184±3.0	342±3.1	171±3.1	146±2.4
30	+	-	0±0.0	257±2.4	172±3.2	147±2.1
30	-	+	183±3.3	339±4.0	169±3.1	146±2.5
30 + Es	+	-	183±2.0	340±2.3	168±3.0	145±2.2
30 + Es	-	+	183±2.0	339±3.0	169±3.5	145±2.5

Incubation mixtures contained the indicated substrate concentrations in µMoles and 50 µl washed human erythrocyte hemolysate (either active or heat-inactivated by incubation in a boiling water bath for 20 minutes as indicated) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0, according to Augustinsson (419). Incubation was carried out at 37°C for the time indicated. Es refers to incubation in the presence of 10^{-5}M eserine sulphate. The values represent the mean of 10 determinations of ester concentration remaining in Klett units ± SD.

Table 2

Hydrolysis of Choline and Carnitine Esters

(Fresh Human Plasma)

Time	Active Enzyme 50 µl	Inact. Enzyme 50 µl	ACh 2 µM	dl-ACCh 2 µM	l-CCh 2 µM	dl-AC 2 µM
min			K.U.	K.U.	K.U.	K.U.
0	+	-	185±1.0	345±3.0	173±1.0	150±0.8
0	-	+	186±0.8	346±2.1	173±0.8	149±1.2
5	+	-	70±1.0	342±1.7	172±1.1	150±0.9
5	-	+	187±1.7	345±2.1	173±0.9	150±1.4
10	+	-	20±0.5	345±0.8	170±2.0	145±1.3
10	-	+	186±1.3	344±1.2	169±1.8	146±2.0
20	+	-	0±0.0	344±3.1	170±2.5	149±0.9
20	-	+	185±1.3	345±1.8	170±2.2	147±2.4
30	+	-	0±0.0	342±3.0	171±1.8	146±1.8
30	-	+	183±1.2	342±2.2	170±0.9	145±2.0
30 + Es	+	-	183±2.0	340±1.8	170±2.0	145±2.1
30 + Es	-	+	183±1.8	341±2.1	170±2.1	146±1.8

Incubation mixtures contained the indicated substrate concentrations in µMoles and 50 µl human plasma (either active or heat-inactivated by incubation in a boiling water bath for 20 minutes as indicated) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0, according to Augustinsson (419). Incubation was carried out at 37°C for the time indicated. Es refers to incubation in the presence of 10^{-5}M eserine sulphate. The values represent the mean of 10 determinations of ester concentration remaining in Klett units ± S.D.

Figure 9

Hydrolysis of Acetylcholine Iodide and dl-Acetylcarnitylcholine Chloride by the Cholinester Hydrolases of Fresh Human Blood

A. Hydrolysis of 0.004M Acetylcholine Iodide

The enzymes were prepared as described in Methods, Part F, 5. The indicated concentrations of substrate in μMoles per total incubation volume of 1.0 ml in 0.1M sodium phosphate buffer, pH 7.0, were incubated with 10 μl fresh enzyme for 30 minutes at 37°C. The amount of ester hydrolyzed was determined by the modified Hestrin method (118). The enzymatic activity was presented in change of optical density in Klett units per 30 minutes. (TABLES XI and XII).

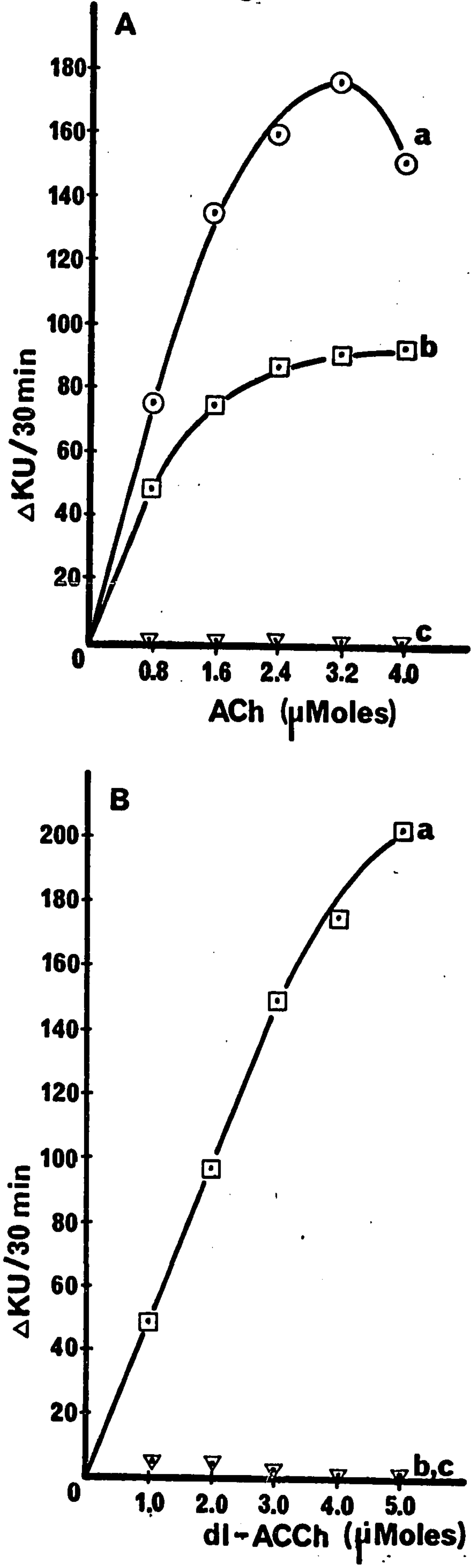
(a) Incubation with 10 μl fresh erythrocyte hemolysate
(b) Incubation with 10 μl fresh human plasma
(c) Incubation with both enzymes in the presence of 10^{-5}M eserine sulphate

B. Hydrolysis of 0.010M dl-Acetylcarnitylcholine Chloride

The same procedures were carried out as above in the presence of the indicated substrate concentrations. (TABLES XIII and XIV).

(a) Incubation with 50 μl fresh erythrocyte hemolysate
(b) Incubation with 50 μl fresh human plasma
(c) Incubation of both enzymes in the presence of 10^{-5}M eserine sulphate

Fig. 9


A
ΔKU/30 min
180
160
140
120
100
80
60
40
20
0
a
b
c
0.8
1.6
2.4
3.2
4.0
ACh (μMoles)
B
ΔKU/30 min
200
180
160
140
120
100
80
60
40
20
0
a
b,c
1.0
2.0
3.0
4.0
5.0
dl-ACCh (μMoles)

hydrolysis of dl-ACCh, on the other hand, appeared to be complete after 20 minutes, when only 25% of the substrate had been hydrolyzed. Since the values presented in the Tables are the mean values for 10 individual readings at each incubation interval, and this 25% total hydrolysis was consistent, the reaction was considered to be complete at this degree of hydrolysis. Using the same incubation conditions, with substrate concentrations of 1.0-3.5 µMoles dl-ACCh/ml, hydrolysis proceeded only until 25% of the substrate had been hydrolyzed.

From Table 2, in which the hydrolytic activity of 50 µl fresh serum was measured, 2 µMoles·ACh were 100% hydrolyzed in 20 minutes. There was, however, no apparent hydrolysis of dl-ACCh, l-CCh or dl-AC by serum cholinesterase. The values in the Table represent the mean of ten determinations at each time. Non-enzymatic controls consisted of incubation in the presence of heat-inactivated enzyme which had been placed in a boiling water bath for 20 minutes and were carried out for every incubation mixture.

Section E. Hydrolysis of Choline and Carnitine Esters by Commercially Purified Cholinester Hydrolases

1. Hydrolysis of Choline and Carnitine Esters by Purified Human Serum Cholinesterase (Schwarz)

The hydrolysis of several esters by a purified preparation of human serum cholinesterase (Schwarz Bioresearch) was examined using the standard incubation methods. Incubation mixtures contained the specified concentrations of substrates, 60 µg pure human serum cholinesterase, either active or heat-inactivated, in a total volume of 1.0 ml 0.1M sodium phosphate buffer, pH 7.0. Non-enzymatic hydrolysis controls consisted of incubating with heat-inactivated enzyme which had been placed in a boiling water bath for 10 minutes.

In Figure 10, the percentage hydrolysis of several compounds with time is presented. It was noted that butyrylcholine (BuCh) was a better substrate for this enzyme than acetylcholine (ACh) although both substrates were hydrolyzed extensively. Succinyldicholine (SDCh), a known substrate of the enzyme, was hydrolyzed at approximately 4% the rate of ACh, in agreement with the findings of Evans *et al.* (100). In this series of five experiments, dl-acetylcarnitylcholine (dl-ACCh) was hydrolyzed at approximately 5% the rate of ACh. This was of interest as no hydrolysis with fresh plasma had been detected. This may have been due to the small amount of enzyme present (plasma cholinesterase represents only approximately 0.01% of the plasma proteins (435)) in the fresh plasma. The finding that there was no hydrolysis in the presence of purified horse serum cholinesterase (see below) however, might be due to species differences.

2. Susceptibility of l-Carnitylcholine Chloride to Cholinester Hydrolase Activity

Figure 10

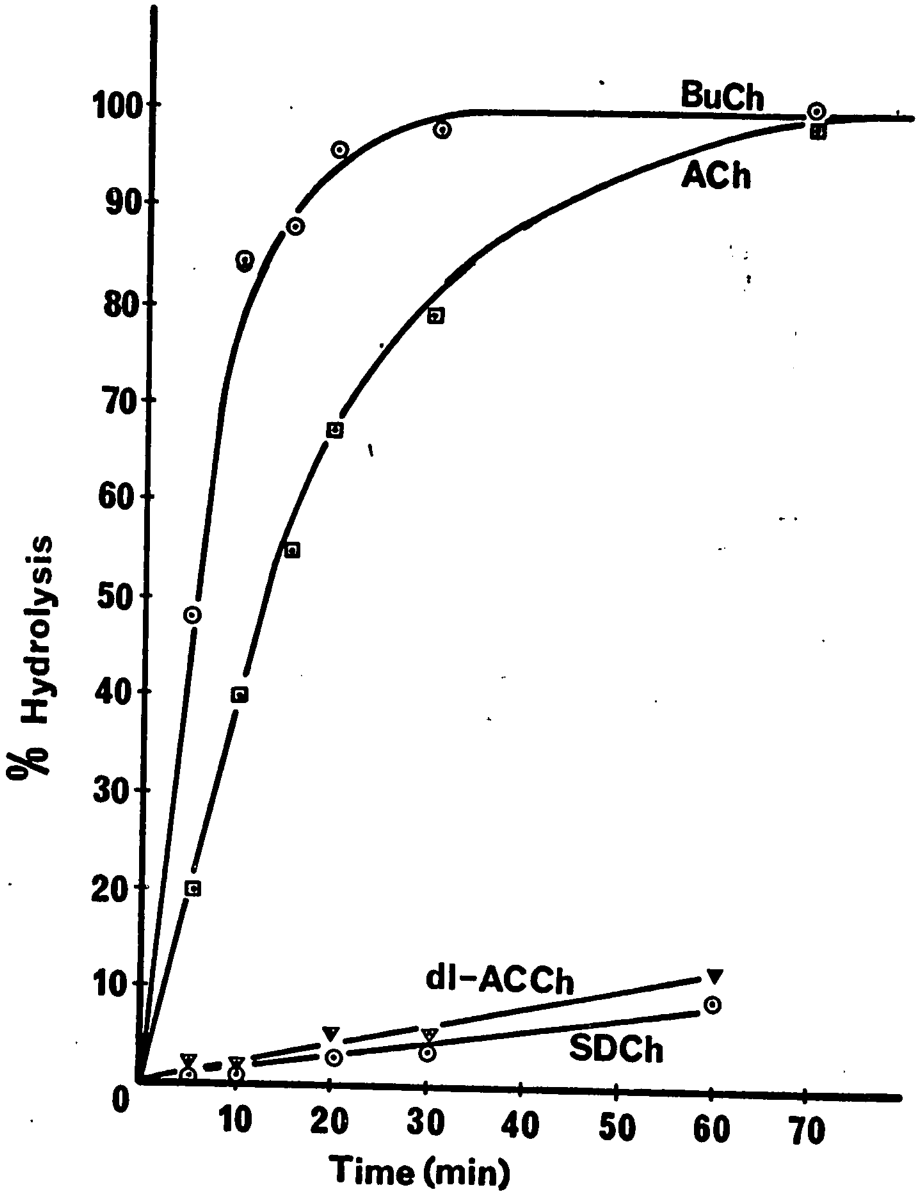
Hydrolysis of Choline and Carnitine Esters by Purified Human Serum Cholinesterase (Schwarz Bioresearch)

The standard method of incubation was employed. The specified concentrations of butyrylcholine iodide (BuCh), acetylcholine iodide (ACh), dl-acetylcarnitylcholine chloride (dl-ACCh), and succinyldicholine chloride (SDCh) were incubated with 60 µg purified human serum cholinesterase in a total volume of 1.0 ml made up with 0.1M sodium phosphate buffer, pH 7.0. Controls for non-enzymatic hydrolysis in the presence of heat-inactivated enzyme were subtracted from the enzymatic values obtained. Incubation was at 37°C for the specified time and the reaction was terminated by the addition of the alkaline hydroxylamine colour reagents. The degree of enzymatic activity was measured by the modified Hestrin method (118) and the values presented are the means of 10 readings at each incubation time. (TABLE XV).

Substrate concentrations:

BuCh	3.0 µMoles/ml	dl-ACCh	2.5 µMoles/ml
ACh	3.0 µMoles/ml	SDCh	2.5 µMoles/ml

Fig.10


BuCh
ACh
dl-ACCh
SDCh
% Hydrolysis
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
Time (min)

In order to determine whether or not either of the purified cholinester hydrolases (bovine erythrocyte, horse serum (Sigma)) could hydrolyze the bis-quaternary compound, l-carnitylcholine (l-CCh), comparison of its hydrolysis to that of acetylcholine iodide (ACh) was carried out with both enzymes. The optimal concentration of ACh iodide, 3 µMoles/ml, was incubated at 37°C with 0.1 mg purified bovine erythrocyte or horse serum cholinesterases in a total volume of 1.0 ml 0.1M sodium phosphate buffer, pH 7.0, for 0 to 30 minutes. Similarly 1,2, and 3 µMoles/ml l-CCh chloride were incubated under identical conditions. The reactions were terminated by the addition of the alkaline hydroxylamine colour reagents and the degree of enzymatic activity was determined colorimetrically employing the modified Hestrin method (118). Controls for non-enzymatic hydrolysis in the presence of heat-inactivated enzyme were carried out for every incubation mixture. Both enzymes, AChE and ChE, completely hydrolyzed ACh whereas, under identical conditions, neither enzyme attacked l-CCh. The mean results of 10 determinations at each incubation time are presented in Tables 3 and 4 and Figure 11.

3. Hydrolysis of dl-Acetylcarnitine by Bovine Erythrocyte Acetylcholinesterase

Attempts to hydrolyze dl-acetylcarnitine (dl-AC) with purified bovine erythrocyte acetylcholinesterase (AChE) (Sigma) failed. Incubation

Table 3

Hydrolysis of l-Carnitylcholine

(Bovine Erythrocyte Acetylcholinesterase)

Substrate conc.			1	2	3
Time	Active Enzyme	Heated Enzyme		(µMoles/ml)	
min	mg	mg		(Readings in Klett units ± S.D.)	
0	0.1	-	88 ± 1.2	175 ± 2.1	266 ± 3.1
0	-	0.1	88 ± 1.3	175 ± 2.1	266 ± 2.5
5	0.1	-	88 + 1.0	175 ± 2.3	266 ± 3.0
5	-	0.1	88 ± 0.8	175 ± 2.1	266 ± 2.8
10	0.1	-	86 ± 1.0	175 ± 3.4	264 ± 2.9
10	-	0.1	88 ± 1.2	175 ± 3.2	266 ± 2.9
20	0.1	-	87 ± 2.1	174 ± 3.2	264 ± 3.2
20	-	0.1	88 ± 1.3	175 ± 3.5	264 ± 3.1
30	0.1	-	88 ± 2.2	172 ± 3.4	262 ± 3.5
30	-	0.1	88 ± 2.2	173 ± 3.1	262 ± 3.4

Incubation mixtures contained the indicated substrate concentrations in µMoles/ml and 0.1 mg purified bovine erythrocyte acetylcholinesterase (Sigma) (either active or heat-inactivated by incubation in a boiling water bath for 10 minutes as indicated) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Incubation was carried out at 37°C for the time indicated. The values represent the mean of 10 determinations of ester concentration remaining in Klett units ± S.D.

Table 4

Hydrolysis of l-Carnitylcholine

(Horse Serum Cholinesterase)

Substrate conc.			1	2	3
Time	Active Enzyme	Heated Enzyme		(μMoles/ml)	
min	mg	mg		Readings in Klett units ± S.D.	
0	0.1	-	87 ± 1.0	156 ± 1.0	252 ± 2.2
0	-	0.1	88 ± 1.2	157 ± 0.5	252 ± 1.0
5	0.1	-	87 ± 1.1	156 ± 0.7	252 ± 2.1
5	-	0.1	87 ± 0.9	157 ± 1.0	252 ± 2.3
10	0.1	-	87 ± 0.8	156 ± 1.1	246 ± 2.2
10	-	0.1	86 ± 1.5	156 ± 1.3	247 ± 2.2
20	0.1	-	86 ± 1.0	153 ± 1.3	246 ± 2.3
20	-	0.1	86 ± 0.9	154 ± 1.2	245 ± 2.5
30	0.1	-	86 ± 1.2	153 ± 1.3	245 ± 2.0
30	-	0.1	86 ± 1.3	154 ± 1.4	245 ± 2.1

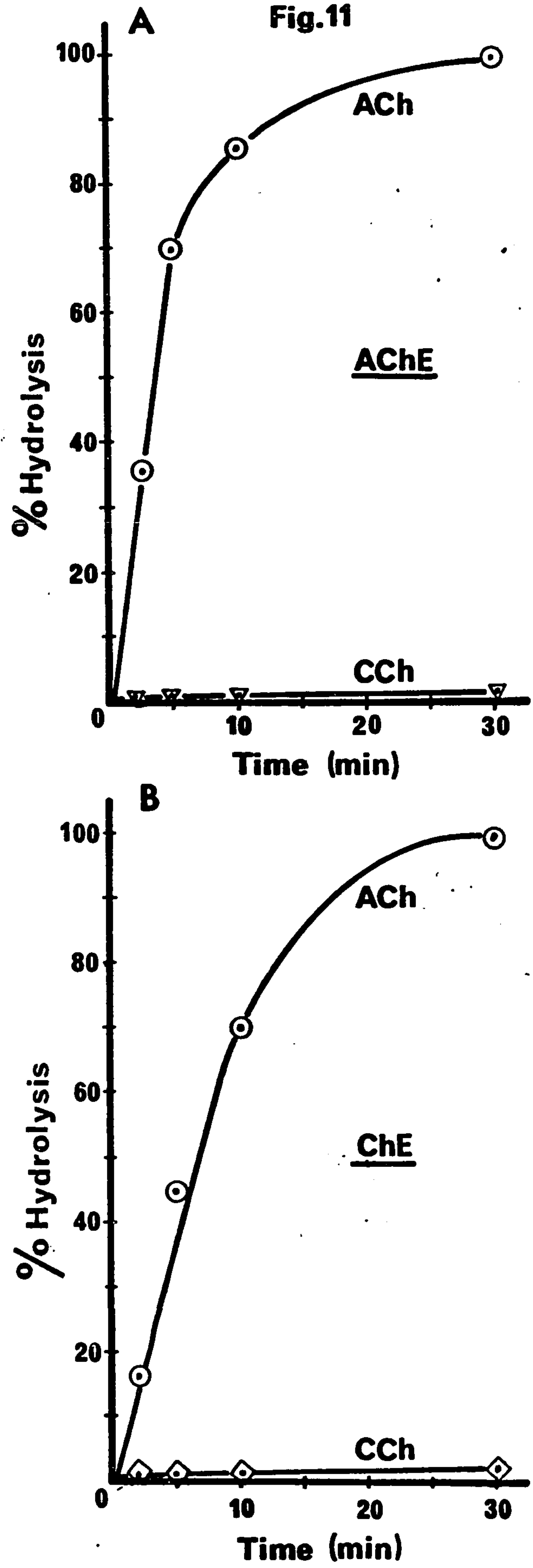
Incubation mixtures contained the indicated substrate concentrations in μMoles/ml and 0.1 mg purified horse serum cholinesterase (either active or heat-inactivated by incubation in a boiling water bath for 10 minutes, as indicated) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Incubation was carried out at 37°C for the time indicated. The values represent the mean of 10 determinations of ester concentration remaining in Klett units ± S.D.

Figure 11

The Susceptibility of Acetylcholine Iodide and l-Carnitylcholine Chloride to Cholinester Hydrolase Actions

Incubation mixtures contained 3 μMoles ACh or 3μMoles l-CCh and either purified bovine erythrocyte acetylcholinesterase (AChE) (Sigma) or the same quantity of purified horse serum cholinesterase (ChE) (Sigma) (0.1 mg in 0.1 ml) made up to a total volume of 1.0 ml with 0.1M sodium phosphate buffer (pH 7.0). Enzymatic activity was measured using the modified Hestrin method (118). Controls for non-enzymatic hydrolysis consisted of incubation in the presence of heat-inactivated enzyme, placed in a boiling water bath for 10 minutes. The values presented are the means of 8 determinations at each incubation time. (TABLE XVI-XVII).

Fig.11


A
100
80
60
40
20
0
10
20
30
% Hydrolysis
Time (min)
ACh
AChE
CCh
B
100
80
60
40
20
0
10
20
30
% Hydrolysis
Time (min)
ACh
ChE
CCh

mixtures contained 3 μMoles dl-AC and 0.05 mg AChE made up to a total volume of 1.0 ml with 0.1M sodium phosphate buffer, pH 7.0. Controls for non-enzymatic hydrolysis consisted of incubation with heat-inactivated AChE, placed in a boiling water bath for 10 minutes. Incubation took place at 37°C for the indicated time intervals and the reaction was terminated by the addition of the alkaline hydroxylamine colour reagents. The amount of ester remaining in each experimental mixture was determined by the modified Hestrin method (118).

The results of three typical experiments are presented in Table 5. The experiment was carried out 8 times with the same result, that is, no hydrolysis of dl-AC by AChE occurred.

4. Hydrolysis of dl-Acetylcarnitylcholine Chloride by Purified Horse Serum Cholinesterase

The possibility that dl-ACCh might be hydrolyzed by serum cholinesterase (ChE) from another species than human, as is the case for its structural isomer SDCh, was examined employing purified horse serum cholinesterase (Sigma). Consistently in 6 experiments no hydrolysis took place at substrate concentrations from 1 to 3 μMoles/ml. Typical results obtained in 4 of the experiments are summarized in Table 6. In every experiment, activity controls consisting of incubation in the presence of 1.0 μMole/ml ACh iodide for 0 to 30 minutes ensured the activity of the enzyme preparation. Controls for non-

Table 5

Hydrolysis of Acetyl-dl-Carnitine

(Bovine Erythrocyte Acetylcholinesterase)

Substrate conc.			1	2	3
Time min	Active Enzyme µg	Heated Enzyme µg	(µMoles/ml) (Readings in Klett units)		
0	50	-	76, 76	151, 151	228, 228
0	-	50	75, 76	151, 151	230, 228
5	50	-	76, 76	151, 150	228, 228
5	-	50	76, 75	151, 151	228, 227
10	50	-	76, 75	151, 150	228, 228
10	-	50	76, 76	151, 151	230, 230
20	50	-	76, 76	151, 151	228, 228
20	-	50	76, 76	151, 151	228, 228
30	50	-	75, 76	151, 152	228, 228
30	-	50	75, 76	152, 151	228, 230

Incubation mixtures contained the indicated substrate concentrations in µMoles/ml and 0.05 mg purified AChE (Sigma) (either active or heat-inactivated by incubation in a boiling water bath for 10 minutes, as indicated) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Incubation was carried out at 37°C for the time indicated. The values represent the ester concentration in Klett units, determined by the modified Hestrin method (118).

Table 6

Hydrolysis of Acetyl-dl-Carnitylcholine

(Horse Serum Cholinesterase)

Time min / Substrate conc.	1.00	2.00 (μMoles/ml)	3.00
	% Hydrolysis		
0	1 ± 1	1 ± 1	0
5	0	1 ± 1	1 ± 1
10	2 ± 2	0	0
15	0	1 ± 1	2 ± 1
20	2 ± 1	1 ± 1	1 ± 1
30	1 ± 1	0 ± 1	1 ± 1
30 + 10^{-5} eserine	0	0	0

Incubation mixtures contained the indicated substrate concentrations in μMoles/ml and 0.1 mg purified horse serum cholinesterase (Sigma) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Incubation was carried out at 37°C for the time indicated and controls for non-enzymatic hydrolysis in the presence of heat-inactivated enzyme were carried out for each incubation time and appropriate corrections made. The values represent the mean of 8 determinations of ester breakdown in % hydrolysis ± S.D.

enzymatic hydrolysis consisted of incubation with heat-inactivated enzyme and appropriate corrections were made. In every case, 10^{-5}M eserine sulphate completely inhibited enzyme activity (TABLE XVIII).

5. Hydrolysis of Acetylcholine Iodide and dl-Acetylcarnitylcholine Chloride by Bovine Erythrocyte Acetylcholinesterase

The course of enzymatic hydrolysis of acetylcholine iodide (ACh) and dl-acetylcarnitylcholine chloride (dl-ACCh) was investigated with purified bovine erythrocyte acetylcholinesterase (AChE) (Sigma).

The standard method of incubation was employed. Incubation mixtures contained varying concentrations of the substrates, 0.1 mg pure AChE, either active or heat-inactivated, made up in a total volume of 1.0 ml 0.1M sodium phosphate buffer, pH 7.0. The reactions were terminated after the specified incubation time at 37°C by the addition of the alkaline hydroxylamine colour reagents and the degree of enzymatic activity was determined by the modified Hestrin method (118).

In Figure 12, the per cent hydrolysis of the two substrates with time is presented. It was noted from Figure 12 that 100% hydrolysis of ACh (1-4 µMoles/ml) took place in 30 minutes. For dl-ACCh the reaction appeared to be complete after only 25% of the ester present had been hydrolyzed. This value of approximately 25% was consistent at substrate concentrations of 1-4 µMoles/ml incubation mixture.

Figure 12

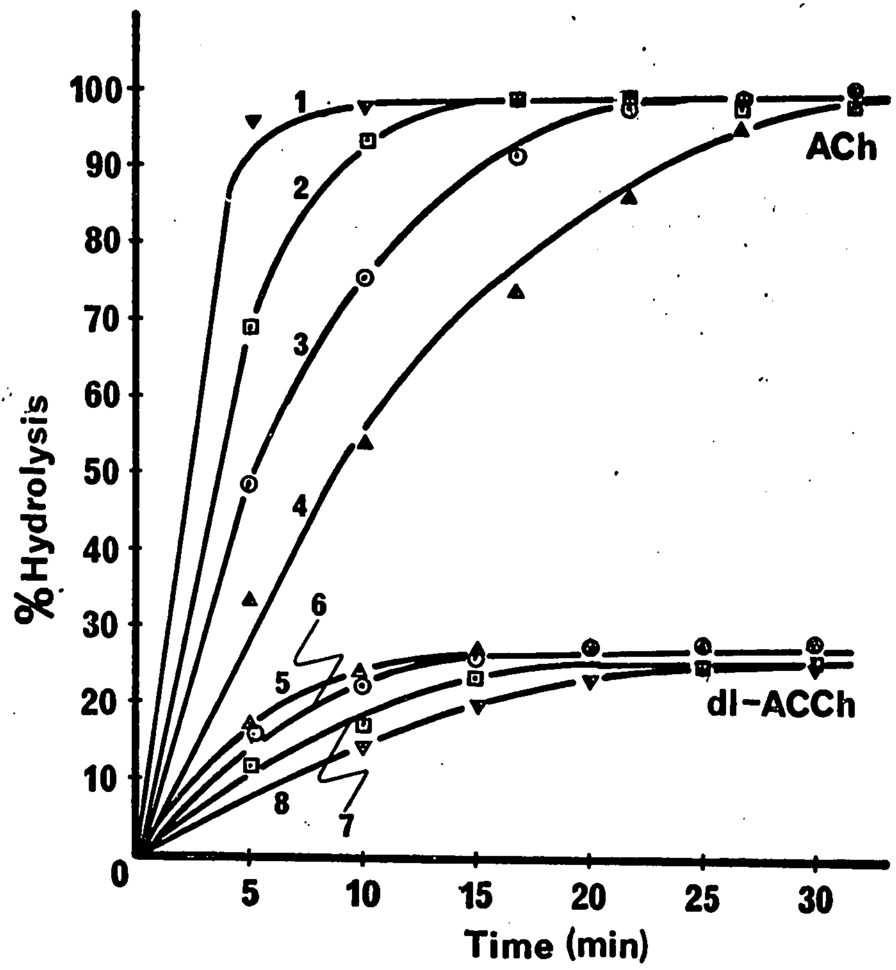
Hydrolysis of Acetylcholine Iodide and dl-Acetylcarnitylcholine Chloride by Purified Bovine Erythrocyte Acetylcholinesterase (Sigma)

The incubation mixtures contained varying amounts of the two substrates as indicated and 0.1 mg bovine erythrocyte acetylcholinesterase (AChE) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Controls for non-enzymatic hydrolysis consisted of incubation with heat-inactivated enzyme. The reaction was terminated by the addition of the alkaline hydroxylamine colour reagents and the degree of activity measured by the modified Hestrin method (118). The results are presented as % substrate hydrolyzed versus time. (TABLE XIX).

The substrate concentrations in μMoles/ml were:

ACh (μMoles/ml)	dl-ACCh (μMoles/ml)
(1) 1.0	(5) 4.0
(2) 2.0	(6) 3.0
(3) 3.0	(7) 2.0
(4) 4.0	(8) 1.0

Fig.12


100
90
80
70
60
50
40
30
20
10
0
% Hydrolysis
5
10
15
20
25
30
Time (min)
1
2
3
4
5
6
7
8
ACh
dl-ACCh

The values are presented in Tables 7 and 8 with the standard deviations for each reading, which represents the mean of 8 determinations. In every case, the non-enzymatic hydrolysis was determined by incubation with heat-inactivated AChE (incubated in a boiling water bath for 10 minutes) and appropriate corrections made. From Tables 7 and 8, it was noted that 10^{-5}M eserine sulphate completely abolished enzymatic activity.

The results from 5 experiments are presented in Figure 13. In these experiments, the numbers of µMoles dl-ACCh hydrolyzed with increasing time of incubation at varying substrate concentrations were investigated. The degree of reaction increased with increasing substrate concentration and a plot of the reciprocals of velocity in µMoles hydrolyzed per minute and substrate mMolar concentration gave a straight line from which an apparent K_m' value of 4×10^{-3}M was determined. The significance of this value is examined in the Discussion.

The possible substrate inhibition by dl-ACCh of the AChE enzyme could not be studied in this system as the optical density readings are out of the range of accuracy above 4 µMoles/ml concentration.

6. Effect of Enzyme Concentration on Hydrolysis of dl-Acetylcarnitylcholine Chloride by Bovine Erythrocyte Acetylcholinesterase (Sigma)

The effect of enzyme concentration upon the velocity of the hydrolysis of dl-ACCh by AChE was investigated at several substrate concentrations.

Table 7

Hydrolysis of Acetylcholine Iodide

(Bovine Erythrocyte Acetylcholinesterase)

Time min	Substrate conc. 1.00	2.00	3.00	4.00
	(μMoles/ml)			
	% Hydrolysis ± S.D.			
0	1 ± 1	3 ± 0	2 ± 1	1 ± 1
5	96 ± 0	69 ± 0	48 ± 2	33 ± 0
10	98 ± 1	94 ± 0	76 ± 1	54 ± 1
15	99 ± 2	99 ± 1	92 ± 1	74 ± 1
20	99 ± 1	99 ± 0	98 ± 1	86 ± 1
25	98 ± 1	99 ± 0	98 ± 1	95 ± 1
30	100 ± 1	99 ± 0	98 ± 1	98 ± 0
30 + 10^{-5}M eserine	0	0	0	0

Incubation mixtures contained the indicated substrate concentrations in μMoles/ml and 0.1 mg purified bovine erythrocyte AChE in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Incubation was carried out at 37°C for the time indicated. The values represent the mean of 12 determinations of ester concentration hydrolyzed in percentage hydrolysis ± S.D. determined by the modified Hestrin method (118).

Table 8

Hydrolysis of Acetyl-dl-Carnitylcholine

(Bovine Erythrocyte Acetylcholinesterase)

Time min	Substrate conc. 0.50	1.00	1.50	2.00 (μMoles/ml)	3.00	4.00
	% Hydrolysis ± S.D.					
0	0	1 ± 0	0.6 ± 0.0	0.5 ± 0.5	1.3 ± 1.0	0 ± 2.5
5	-	17 ± 4	14.7 ± 0.6	15.0 ± 1.5	12.7 ± 0.6	8.2 ± 5.0
10	10 ± 0	24 ± 2	22.0 ± 0.6	17.0 ± 0.5	15.0 ± 2.3	15.5 ± 1.2
15	24 ± 2	26 ± 1	26.0 ± 2.0	23.6 ± 0.0	20.7 ± 0.0	27.5 ± 0.8
20	28 ± 0	28 ± 0	26.6 ± 0.6	24.0 ± 0.0	23.0 ± 0.0	-
25	28 ± 2	28 ± 4	26.6 ± 2.0	24.5 ± 1.5	25.0 ± 1.0	27.0 ± 2.5
30	28 ± 2	28 ± 1	26.0 ± 2.0	25.5 ± 0.0	25.6 ± 1.6	27.0 ± 2.5
30 + 10^{-5}M eserine	0	0	0	0	0	0

Incubation mixtures contained the indicated substrate concentrations in μMoles/ml and 0.1 mg purified bovine erythrocyte AChE (Sigma) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Incubation was carried out at 37°C for the time indicated. The values represent the mean of 12 determinations of ester breakdown in % hydrolysis determined by the modified Hestrin method (118).

Figure 13

Hydrolysis of dl-Acetylcarnitylcholine Chloride by Acetylcholinesterase

A. Effect of Substrate Concentration on AChE Activity

The effect of varying the concentration of dl-acetylcarnitylcholine chloride (dl-ACCh) from 1 to 3 µMoles/ml incubation mixture on the degree of hydrolysis with time is presented. Standard conditions were employed. Enzyme concentration was 0.1 mg bovine erythrocyte acetylcholinesterase (Sigma) (AChE) in a total incubation mixture volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. The mixtures were incubated at 37°C for the indicated times and the reaction was terminated by the addition of the alkaline hydroxylamine colour reagents. The degree of hydrolysis was determined by the modified Hestrin method (118). Controls for non-enzymatic hydrolysis consisted of incubation with heat-inactivated enzyme and appropriate corrections were made. The results represent the mean values of 5 experiments. (TABLE XIX).

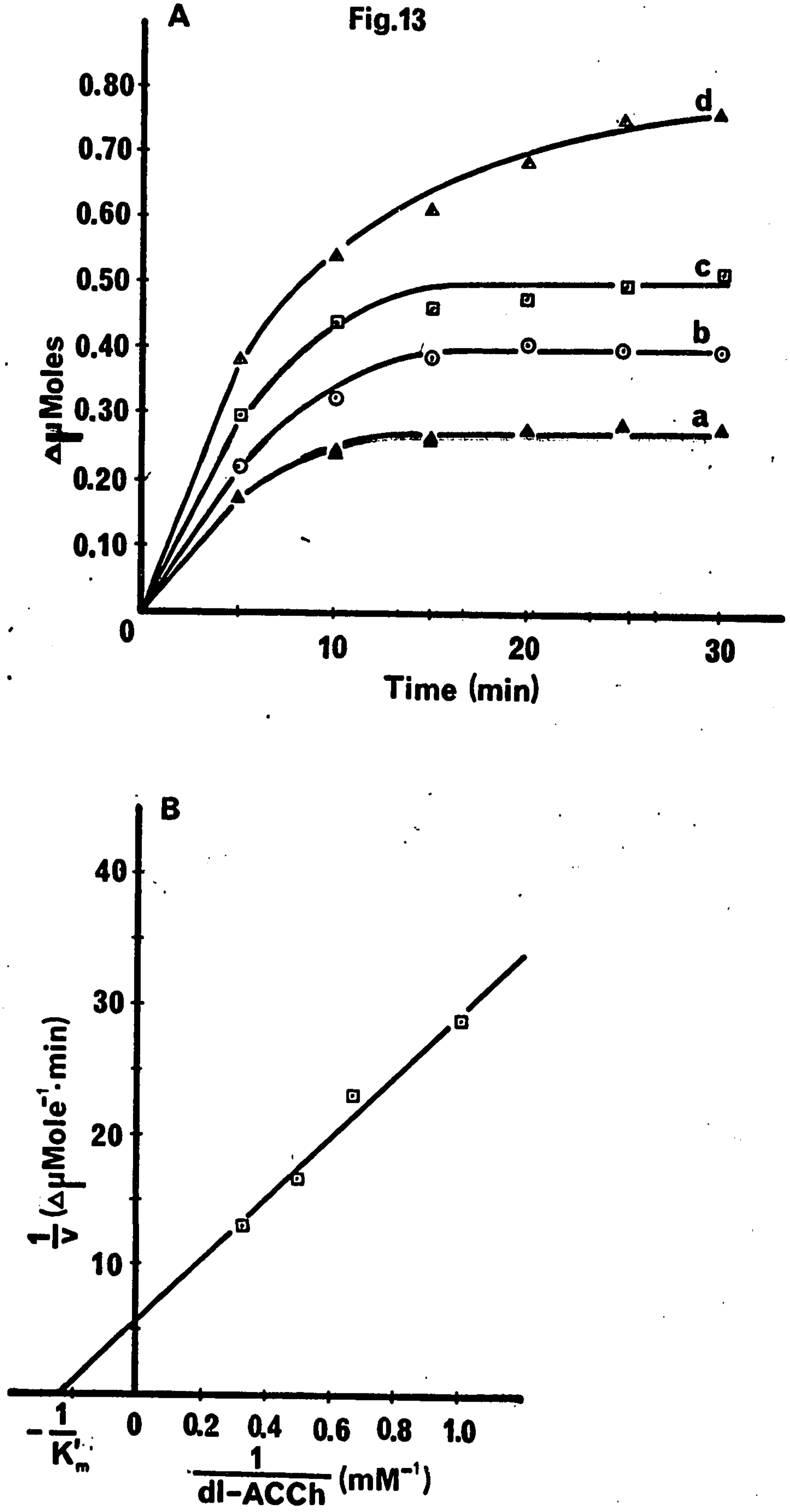
dl-ACCh concentrations (µMoles/ml)

(a) 1.0
(b) 1.5
(c) 2.0
(d) 3.0

B. Lineweaver-Burk Plot

The same data were plotted according to Lineweaver and Burk, having calculated initial velocities in µMoles substrate hydrolyzed per minute. The apparent K_m' value obtained graphically was 4×10^{-3}M.

Fig.13


A
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
ΔμMoles
d
c
b
a
10
20
30
Time (min)
B
40
30
20
10
$\frac{1}{v}$ (ΔμMole^{-1}·min)
$-\frac{1}{K'_m}$
0
0.2
0.4
0.6
0.8
1.0
$\frac{1}{\text{dl-ACCh}}$ (mM^{-1})

The reaction velocity increased with increasing enzyme concentration although the relationship was not always linear (Figure 14,A). Of particular interest, however, was the consistent finding shown in Figure 14, A, that the reaction appeared to be terminated when only 25% of the ester bonds in solution were cleaved. As the standard enzyme assay by the modified Hestrin method (118) was employed in these experiments, the total ester bonds in solution were determined and the amount of hydrolysis was calculated by the decrease of ester concentration in the incubation mixtures.

A different commercial preparation of AChE was examined and gave essentially the same results (Figure 14,B). The velocities of reaction were presented as the number of μMoles hydrolyzed per 10 minutes or the decrease in optical density in Klett units per 15 minutes, the values representing the means of 10 determinations.

7. The pH Dependence of the Hydrolysis of dl-Acetylcarnitylcholine Chloride by Bovine Erythrocyte Acetylcholinesterase

It was of interest to determine whether or not acetylcholinesterase (AChE) exhibited the same pH dependence for the hydrolysis of dl-ACCh as it did for ACh. In these experiments the method of Bergmann et al. (460) was employed. For the pH range 5.5 to 8.5 the buffer was a sodium phosphate buffer (0.1M). For higher pH's from 8.5 to 10.0 a 0.1M solution of sodium borate buffer was employed. A 0.1M sodium acetate buffer gave

Figure 14

Effect of Enzyme Concentration on Hydrolysis of dl-Acetylcarnitylcholine Chloride by Bovine Erythrocyte Acetylcholinesterase

A. Incubation mixtures contained in a total volume of 1.0 ml 0.1M sodium phosphate buffer, pH 7.0, the specified amounts of enzyme (5 mg AChE (Sigma) /25 ml phosphate buffer) and the concentrations of dl-ACCh indicated below. Incubation was carried out at 37°C for 10 minutes and the reaction was terminated by addition of the alkaline hydroxylamine colour reagents. The amount of ester hydrolyzed was determined by the modified Hestrin method (118). Controls for non-enzymatic hydrolysis consisted of incubation in the presence of heat-inactivated enzyme, incubated in a boiling water bath for 10 minutes. (Reaction velocity is in μMoles hydrolyzed/10 min). (TABLE XX).

dl-ACCh concentrations:

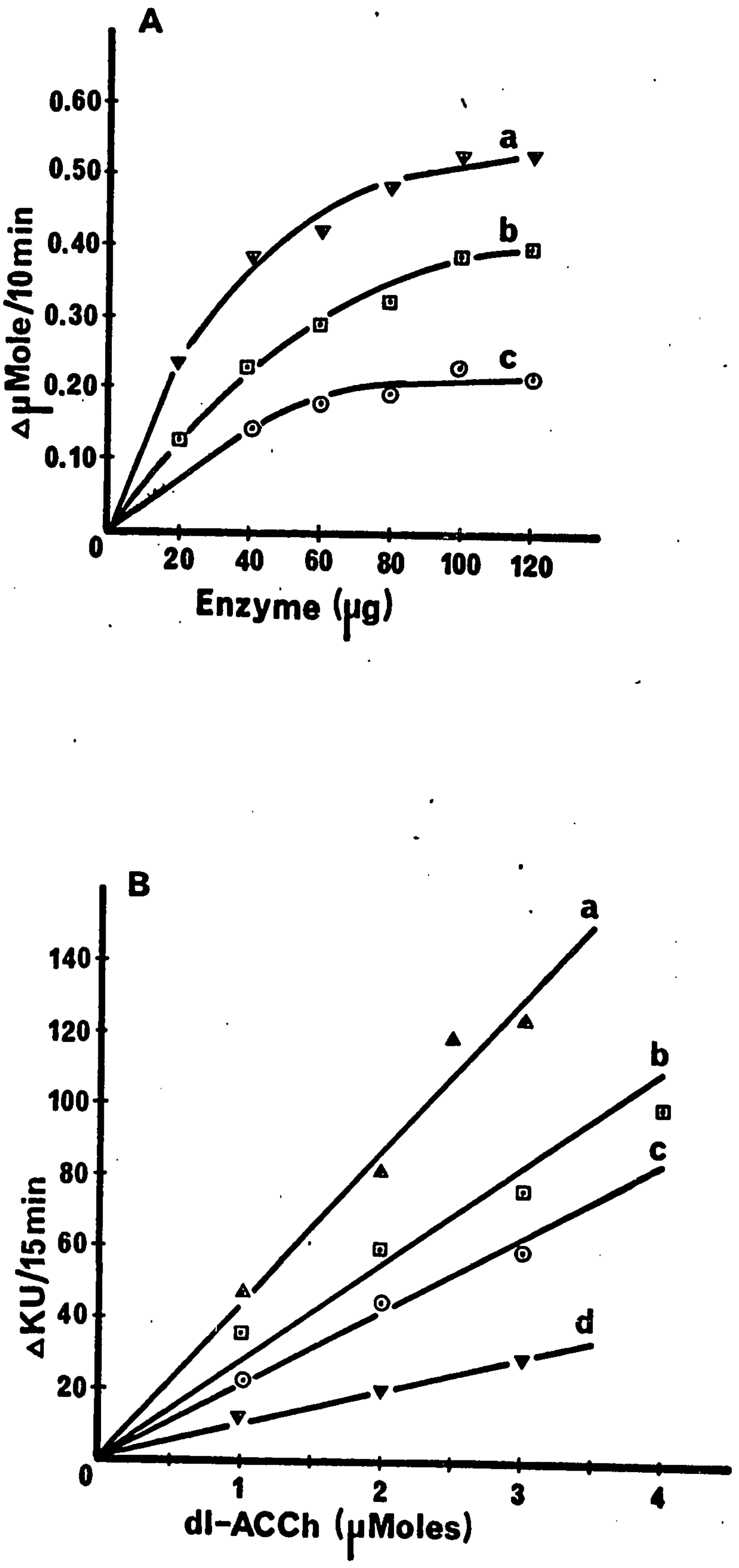
(a) 2.2 μMoles/ml
(b) 1.7 μMoles/ml
(c) 0.99 μMoles/ml

B. The same procedure was employed using a freshly supplied preparation of bovine erythrocyte AChE (Sigma). (TABLE XXI).

Enzyme concentrations:

(a) 80 μg/ml
(b) 60 μg/ml
(c) 40 μg/ml
(d) 20 μg/ml

Fig.14


A
0.60
0.50
0.40
0.30
0.20
0.10
0
20
40
60
80
100
120
Δ μMole/10min
Enzyme (μg)
a
b
c
B
140
120
100
80
60
40
20
0
1
2
3
4
ΔKU/15min
dl-ACCh (μMoles)
a
b
c
d

a pH of 4.1 and a mixture of sodium bicarbonate and sodium carbonate gave a desired pH of 10.6. In every case, the ionic strength was approximately 0.1.

The enzyme employed was 0.1 mg purified bovine erythrocyte AChE (Sigma) (1 mg AChE/ml 0.1M sodium phosphate buffer, pH 7.2). Controls consisted of the addition of enzyme, heat-inactivated by incubating in a boiling water bath for 10 minutes.

The incubation mixtures contained 1.33 μMoles dl-ACCh in 0.1 ml, 0.1 ml 0.1% AChE solution and 0.8 ml of the appropriate buffer. The enzymatic reaction was started by the addition of substrate, and terminated after 1,2,3 and 5 minute intervals by the addition of the alkaline hydroxylamine colour reagents.

The pH of each incubation mixture was determined prior to incubation in order to allow for any effect of the enzyme protein buffering capacity. The degree of hydrolysis was determined by the modified Hestrin method (118) and the initial reaction velocities were calculated at each pH.

The results of a typical experiment at pH 6.27 are presented in Table 9. Each incubation mixture contained 1.33 μMoles dl-ACCh solution made up in 0.1 ml distilled water, 0.1 ml 0.1% AChE made up in 0.1M sodium phosphate buffer, pH 7.2, and 0.8 ml 0.1M sodium phosphate buffer, pH 6.0 to give a total volume of 1.0 ml. The final pH of the mixture was 6.27. Omission of the substrate from the incubation mixture constituted the

Table 9

Hydrolysis of dl-Acetylcarnitylcholine by Acetylcholinesterase (Sigma)

pH 6.27

Time min	Active enzyme (0.1 mg)	Inact. enzyme	O.D. (Klett units)	Average ΔO.D.	% Hydrolysis	%/min	μMoles Hydrolysis	ΔμMoles
0	+	-	232;230	0	0		0	
0	-	+	229;230		0		0	
1	+	-	214;216	8	6.5	3.5	0.087	0.047
1	-	+	222;224		3.0		0.040	
2	+	-	201;201	17	12.6	7.5	0.168	0.099
2	-	+	218;218		5.1		0.069	
3	+	-	192;192	26	16.5	11.4	0.220	0.151
3	-	+	218;218		5.1		0.069	
5	+	-	181;182	35	21.3	15.3	0.284	0.203
5	-	+	216;216		6.0		0.081	

Incubation mixtures contained 1.33 μMoles dl-ACCh, 0.1 mg bovine erythrocyte AChE made up in 0.1 ml 0.1M sodium phosphate buffer, pH 7.2, and 0.8 ml 0.1M sodium phosphate buffer, pH 6.0,to a total volume of 1.0 ml. The final mixture pH was 6.27. Incubation was carried out at 37°C for the indicated times and the reactions terminated by the addition of the alkaline hydroxylamine colour reagents. The amounts of ester remaining were determined by the modified Hestrin method (118) in Klett units.

reaction blank. Controls for non-enzymatic hydrolysis of the substrate were obtained by incubation with inactivated enzyme which had been heated in a boiling water bath for 10 minutes. The reaction was terminated after the indicated incubation time by addition of alkaline hydroxylamine and the mixtures were analyzed colorimetrically by the Hestrin method modified by Friedman and Fraenkel (118).

The initial reaction velocity in μMoles dl-ACCh hydrolyzed per minute at a range of pH's is presented in Figure 15. The optimum pH range was from 7 to 8 as was found for the hydrolysis of ACh by the same enzyme (460). The values presented in the Figure are the mean values from 3 experiments carried out in duplicate at each pH.

8. Effect of Temperature on Hydrolysis by Acetylcholinesterase

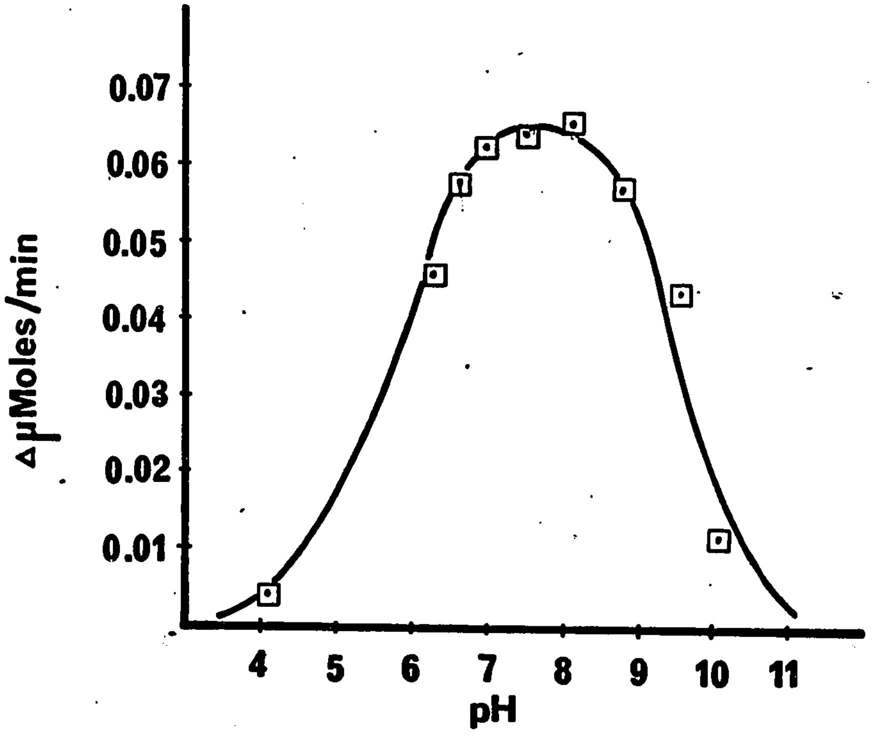
When one enzyme acts on several substances, the same activation energy is frequently obtained for all as the activation energy appears to be more characteristic of the enzyme than of the substrate. Ideally log V_{max} should be plotted against the reciprocal of the absolute temperature. V_{max} could be obtained by extrapolation from a Lineweaver-Burke curve at each temperature. This required a large quantity of substrate, which was unavailable, and therefore initial velocities at the optimum substrate concentration were determined.

Figure 15

The pH Dependence of dl-Acetylcarnitylcholine Chloride Hydrolysis by Purified Bovine Erythrocyte Acetylcholinesterase

The complete reaction mixture contained 1.33 µMoles dl-ACCh solution made up in 0.1 ml distilled water, 0.1 ml 0.1% AChE solution made up in 0.1M sodium phosphate buffer, pH 7.2, and 0.8 ml of the appropriate buffers to give a final incubation volume of 1.0 ml. Omission of the substrate from the complete mixture constituted the reaction blank. Controls for non-enzymatic hydrolysis were obtained by incubating in the presence of heat-inactivated enzyme. The reaction was terminated by addition of alkaline hydroxylamine and mixtures were analyzed colorimetrically by the modified Hestrin method (118). The results at each pH were plotted to determine the initial reaction velocity in µMoles dl-ACCh hydrolyzed/minute. The values presented in the Figure are the mean values from 3 experiments at each pH. The maximum variation from the mean was only 2.2%. (TABLE XXII).

Fig.15



(i) Temperature Dependence of Acetylcholine Hydrolysis by Acetylcholinesterase

The enzyme was treated at various temperatures for 5 minute intervals. Pure bovine erythrocyte acetylcholinesterase (Sigma) (AChE), 2.0 mg in M/15 sodium phosphate buffer (pH 7.2) in a total volume of 2.0 ml, was heated at the indicated temperature, cooled, and 0.1 ml aliquots assayed for hydrolytic activity by the standard modified Hestrin method (118) using 3.0 μMoles ACh iodide as substrate in a total incubation volume of 1.0 ml. Enzymatic activity was determined at 1,2,5 and 8 minute intervals at 37°C.

The enzyme retained full activity at 45°C, but inactivation started at 52°C and at 75°C the activity was lost after 5 minutes. Interpolation of the curve revealed that 50% of the enzyme could be expected to be inactivated when heated for 5 minutes at 65°C (Figure 16,A). When the log of the initial velocity of the enzyme reaction (v = μMoles ACh hydrolyzed per minute), measured at less than 45°C, was plotted against the reciprocal of the absolute temperature (1/T), a straight line was obtained (Figure 16,C), in agreement with the Arrhenius equation:

$$\log k = - \frac{E}{2.303\ R} \times \frac{1}{T} + \log S$$

where E is the heat of activation, R is the gas constant, and S is a constant. From the slope of the line, a value of 4,211 cal/mole was obtained.

(ii) Temperature Dependence of dl-Acetylcarnitylcholine Hydrolysis by Acetylcholinesterase

Figure 16

The Effect of Temperature on the Hydrolysis of Acetylcholine Iodide by Bovine Erythrocyte Acetylcholinesterase (Sigma)

A. Inactivation of AChE at Elevated Temperatures

Standard mixtures containing 0.1 mg purified AChE were preincubated at the indicated temperature for 5 minutes, and the activity assayed by the modified Hestrin method (118) at 37°C employing 3 μMoles ACh per ml as substrate.

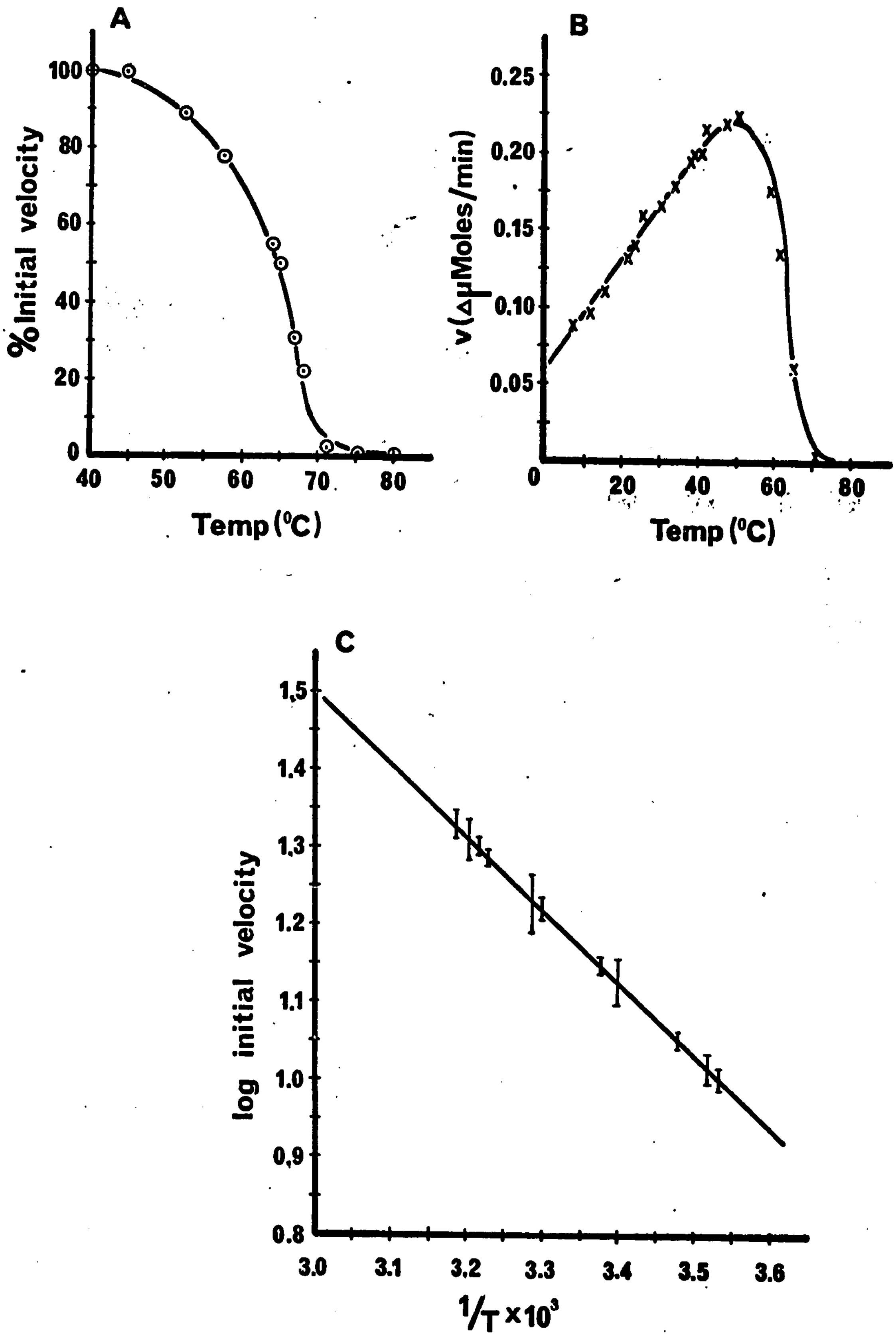
B. Effect of Temperature on Initial Reaction Velocity

The standard incubation procedure was followed. ACh hydrolysis by AChE was determined at the indicated incubation temperatures. The reaction velocity increased to 47°C. Initial velocities were expressed as μMoles ACh hydrolyzed per minute, and the temperature was in degrees centrigrade.

C. The Arrhenius Plot of ACh Hydrolysis by AChE

Standard mixtures containing 0.1 mg pure AChE were employed. Each mixture was incubated at the indicated temperature for 5 minutes and the activity assayed by the modified Hestrin method (118). The initial velocity is expressed as μMoles ACh hydrolyzed per 10 minutes, and T is the absolute temperature. The straight line was calculated by the least squares regression line method employing the results of 5 experiments and the standard deviations from the line at each temperature are presented. (TABLE XXIII).

Fig.16


A
%Initial velocity
100
80
60
40
20
0
40
50
60
70
80
Temp (°C)
B
v (ΔμMoles/min)
0.25
0.20
0.15
0.10
0.05
0
20
40
60
80
Temp (°C)
C
log initial velocity
1,5
1.4
1,3
1.2
1.1
1.0
0,9
0.8
3.0
3.1
3,2
3.3
3.4
3.5
3.6
1/T × 10³

Having established the reliability of the system with ACh, the determination of the temperature dependence of dl-ACCh hydrolysis by bovine erythrocyte acetylcholinesterase (Sigma) (AChE) was studied. As expected, the activation energy was similar to that of ACh and was very much lower than that reported for ordinary enzymes (432).

The enzyme was treated at various temperatures for 5 minute intervals. Pure AChE (Sigma), 2.0 mg in M/15 sodium phosphate buffer, pH 7.2, in a total volume of 2.0 ml, was heated at the indicated temperature, cooled, and 0.1 ml aliquots were assayed for hydrolytic activity by the standard modified Hestrin method (118) using 2.0 μMoles dl-ACCh as substrate in a total volume of 1.0 ml. The enzyme retained full activity at 45°C, inactivation began at 47°C and at 70°C the activity was gone in 5 minutes. From the curve (Figure 17,A), 50% of the enzyme was inactivated at 60°C in 5 minutes. The maximum activity was from 38-42°C. dl-ACCh hydrolysis by AChE was dependent on temperature, the velocity increasing with temperature to 45°C. Above this temperature, the activity decreased abruptly (Figure 17,B). When the log of initial velocity of the enzyme reaction (v = μMoles dl-ACCh hydrolyzed per minute), measured at less that 45°C, was plotted against the reciprocal of the absolute temperature (1/T), a straight line was obtained (Figure 17,C), in agreement with the Arrhenius equation:

$$\log k = - \frac{E}{2.303\ R} \times \frac{1}{T} + \log S$$

where E is the heat of activation, R is the gas constant, and S is a constant.

Figure 17

The Effect of Temperature on the Hydrolysis of dl-Acetylcarnitylcholine Chloride by Bovine Erythrocyte Acetylcholinesterase

A. Inactivation of AChE at Elevated Temperatures

Standard mixtures containing 0.1 mg purified AChE (Sigma) were preincubated at the indicated temperatures for 5 minutes, and the residual activity assayed by the standard modified Hestrin method (118) employing 2 µMoles dl-ACCh per ml as substrate.

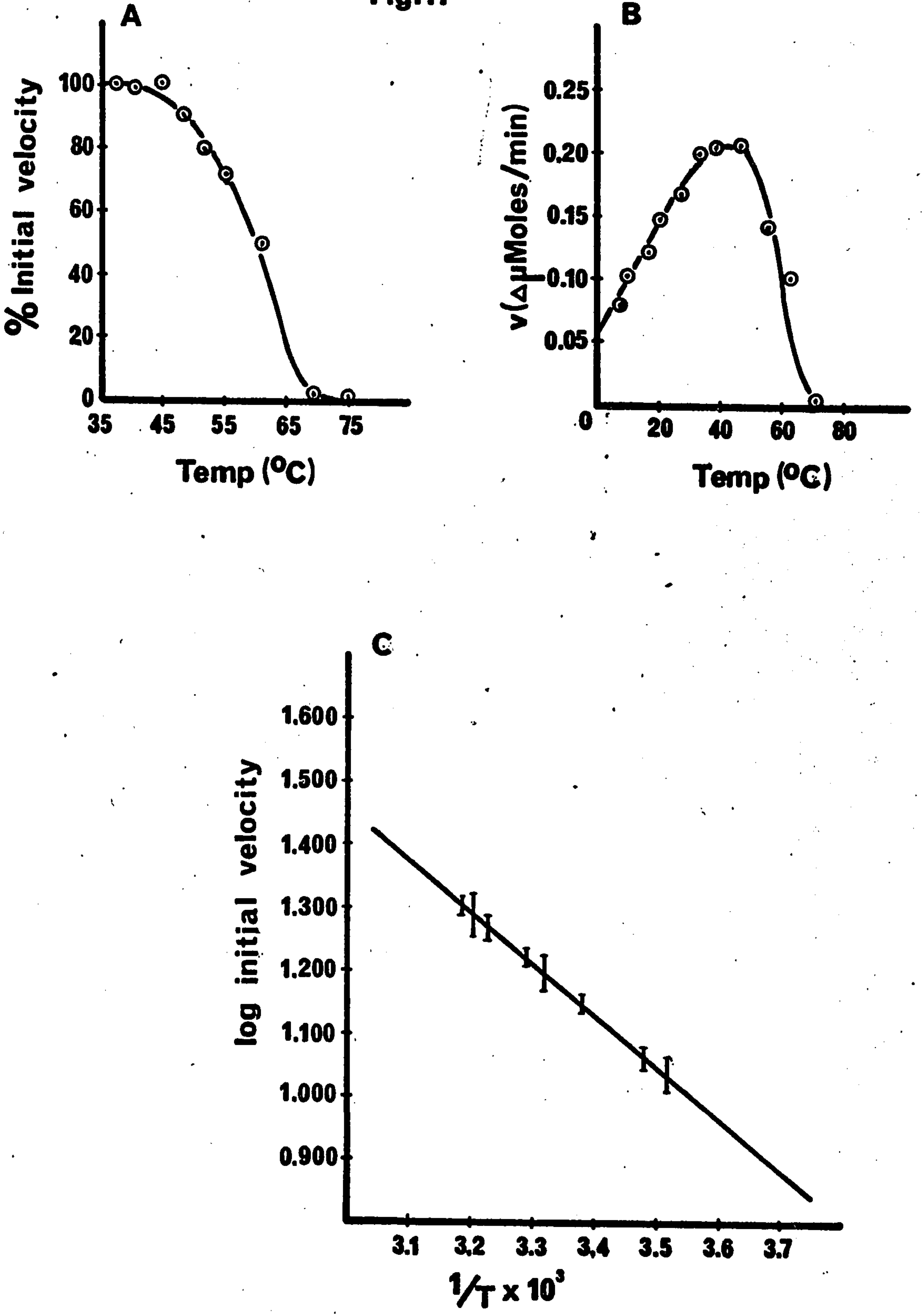
B. Effect of Temperature on Initial Reaction Velocity

The standard incubation procedure was employed. dl-ACCh hydrolysis by AChE was determined at the indicated incubation temperatures. Initial velocities were expressed as µMoles dl-ACCh hydrolyzed per minute, and the temperature was in degrees centrigrade.

C. The Arrhenius Plot of dl-ACCh Hydrolysis by AChE

Standard mixtures containing 0.1 mg pure AChE were employed. Each mixture was incubated at the indicated temperature for 5 minutes, and the activity assayed by the modified Hestrin method. The initial velocity was expressed as µMoles dl-ACCh hydrolyzed per 10 minutes, and T was the absolute temperature. The straight line was calculated by the method of least squares employing the results of 4 experiments and the standard deviations from the line at each temperature are presented. (TABLE XXIV).

Fig.17


A
% Initial velocity
100
80
60
40
20
0
35
45
55
65
75
Temp (°C)
B
v(ΔμMoles/min)
0.25
0.20
0.15
0.10
0.05
0
20
40
60
80
Temp (°C)
C
log initial velocity
1.600
1.500
1.400
1.300
1.200
1.100
1.000
0.900
3.1
3.2
3.3
3.4
3.5
3.6
3.7
1/T x 10³

From the slope of the line, a value of 3,800 cal/mole was obtained.

Thus the enzymic hydrolysis of the two substrates exhibited similar energies of activation which agreed fairly well with the accepted values for erythrocyte AChE. This low energy of activation has been associated with a very large entropic effect for the hydrolysis of ACh by eel AChE by Wilson and Cabib (427). The entropy changes involved in the interaction of dl-ACCh and AChE remain to be investigated.

Section F. The "Anticholinesterase" Activity of Choline and Carnitine Esters

The inhibition of the cholinester hydrolases by a series of choline and carnitine esters was investigated.

1. Anticholinesterase Activity of l-Carnitylcholine Assayed Colorimetrically by the Modified Hestrin Method (118)

The possibility of employing the standard modified Hestrin method (118) for assaying the inhibitory effects of related esters on the hydrolysis of acetylcholine by acetylcholinesterase (AChE) was examined. The results of a typical experiment on the inhibition of ACh hydrolysis by synthetic l-carnitylcholine (l-CCh) are presented in Table 10.

In the absence of l-CCh, 2 μMoles/ml ACh were hydrolyzed rapidly in a linear fashion. In 4 minutes, the decrease in colour due to ester

Table 10

Hydrolysis of Acetylcholine in the Presence of l-Carnitylcholine

(Bovine Erythrocyte Acetylcholinesterase)

Inhibitor conc.						
Time	Active Enzyme	Inact. Enzyme	0	1	2	3
				(µMoles/ml)		
min	(60 µg)			(Readings in Klett units)		
0	+	-	187;187	282;282	365;362	455;460
0	-	+	185;185	282;282	365;365	460;460
1	+	-	170;174	266;266	353;352	449;450
1	-	+	187;187	282;280	360;360	450;450
2	+	-	157;157	257;258	340;345	445;445
2	-	+	187;187	280;282	360;360	450;450
3	+	-	140;142	246;246	330;332	435;435
3	-	+	188;187	276;276	355;355	445;445
4	+	-	130;130	240;240	320;320	425;425
4	-	+	187;186	274;274	355;355	440;440

The substrate concentration was 2 µMoles/ml in all incubation mixtures. Incubation was carried out at 37°C and the total volume was 1.0 ml made up with 0.8 ml 0.1M sodium phosphate buffer, pH 7.0. The reaction was started by the addition of 60 µg enzyme to the incubation mixtures which had been equilibrated to 37°C. Controls consisted of incubation in the presence of heat-inactivated AChE which had been placed in a boiling water bath for 10 minutes. Enzymatic activity was determined by the modified Hestrin method (118).

was 57 Klett units, or the equivalent of 0.62 μMoles ACh hydrolyzed.

The presence in the incubation mixture of equal concentrations of ACh and I-CCh, that is, 2 μMoles of each, reduced the decrease in ester reading to 35 Klett units, or the equivalent of 0.36 μMoles ACh hydrolyzed.

Since the enzyme had no effect upon the colour produced by I-CCh at any of the concentrations studied, that is, AChE did not hydrolyze I-CCh, the decrease in hydrolysis could only be due to a change in the degree of hydrolysis of ACh. Thus, AChE hydrolysis of ACh was inhibited by I-CCh.

This method, however, could not conveniently be employed in the study of the inhibitory effects of those esters which were themselves hydrolyzed by the enzyme. In such a case, the sum of substrate and inhibitor hydrolysis would be determined by this method. It was obvious, also, from Table 9, that the addition of more than 3 μMoles ester inhibitor (or even less for the diester inhibitors) gave an initial optical density greater than 450 Klett units, and was thus above the range of accuracy and dependability of the modified Hestrin method. For these reasons, the modified Hestrin method was not generally employed for the investigation of "anticholinesterase" activity.

2. Anticholinesterase Activity of Choline and Carnitine Esters Assayed Colorimetrically by the Thiocholine Method

The inhibitory activity of a series of choline and carnitine esters on the hydrolysis of acetylthiocholine iodide (AcTCh) by bovine erythrocyte acetylcholinesterase (AChE) (Sigma) was investigated using the Rubin (497) modification of the method of McOsker et al. (479).

The assay system was studied first in order to determine its reliability and reproducibility. The effect of varying enzyme concentration on the activity measured by the appearance of sulphydryl groups (Δ O.D.) for a 10 minute incubation under the standard conditions is given in Figure 18,A, for a substrate concentration of 1.5×10^{-3}M AcTCh. The relationship was linear to an enzyme concentration of 0.1 mg AChE.

The time course of the reaction is presented in Figure 18,B, and was linear for 10 minutes in the presence of 0.1 mg AChE and 1.5×10^{-3}M AcTCh.

The effect of varying the substrate concentration on the initial velocity of reaction is presented in Figure 19,A. Maximum velocity was obtained with 1.5×10^{-3}M AcTCh in the presence of 0.1 mg AChE. The data were recalculated and presented in the form of a Lineweaver-Burke reciprocal plot in Figure 19,B, from which an apparent K_m' value of 3×10^{-4}M was determined.

A series of choline and carnitine esters were studied to determine their inhibitory effect on AChE hydrolysis of AcTCh. The incubation mixtures consisted of varying concentrations of substrate, inhibitor, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated

Figure 18

A. Effect of Enzyme Concentration on the Enzymatic Hydrolysis of Acetylthiocholine Iodide

Bovine erythrocyte AChE (Sigma) (1mg/ml) was incubated at 37°C for 10 minutes with 1.5 x 10^{-3}M AcTCh. Velocity of the reaction is presented in change of optical density (Δ O.D.) at 520 mμ/10 minutes. The mean values from 8 determinations are presented. (TABLE XXV).

B. Effect of Incubation Time on the Enzymatic Hydrolysis of Acetylthiocholine Iodide

Bovine erythrocyte AChE (Sigma) (0.1 mg in 0.1 ml) was incubated at 37°C with 1.5 x 10^{-3}M AcTCh. Activity is presented in change of optical density (Δ O.D.) at 520 mμ. The mean values from 8 determinations are presented. (TABLE XXVI).

Fig.18

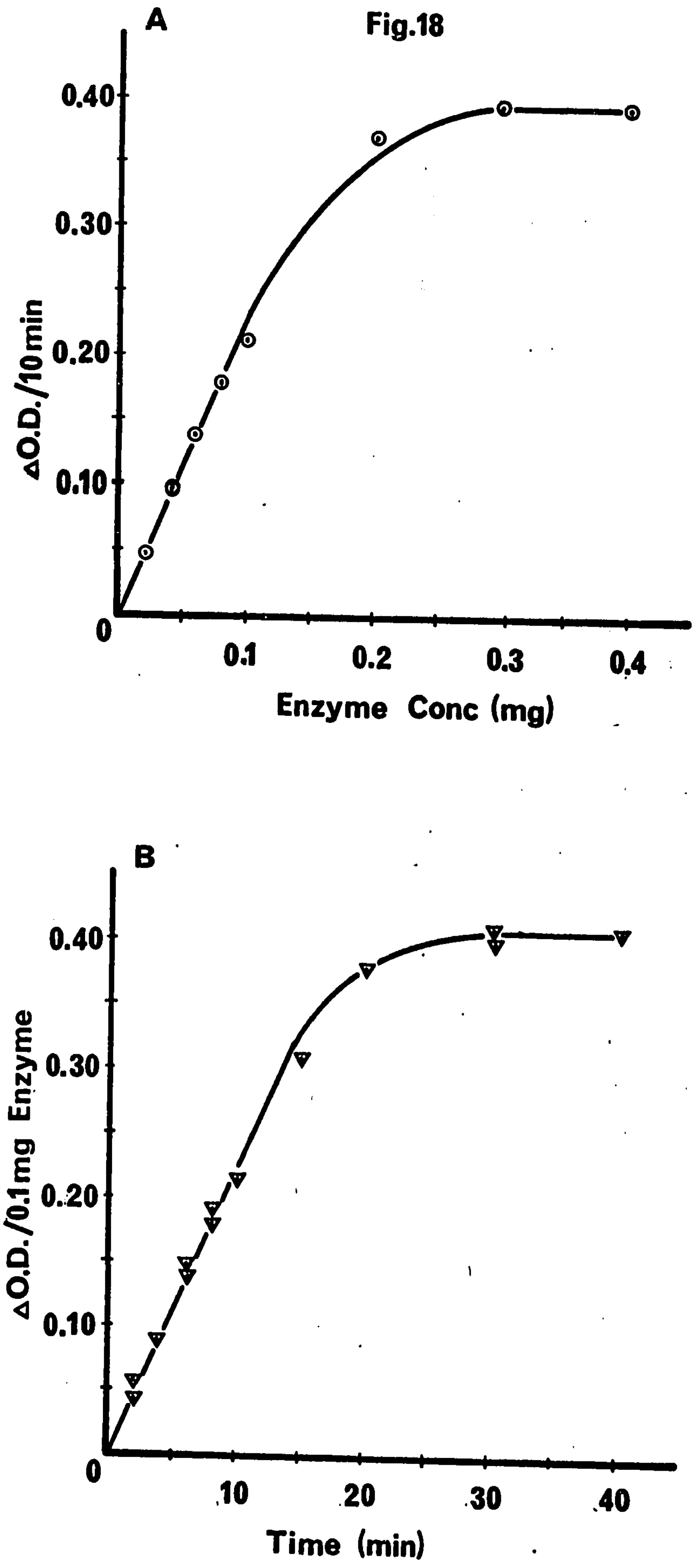

A
ΔO.D./10min
0.40
0.30
0.20
0.10
0
0.1
0.2
0.3
0.4
Enzyme Conc (mg)
B
ΔO.D./0.1mg Enzyme
0.40
0.30
0.20
0.10
0
10
20
30
40
Time (min)

Figure 19

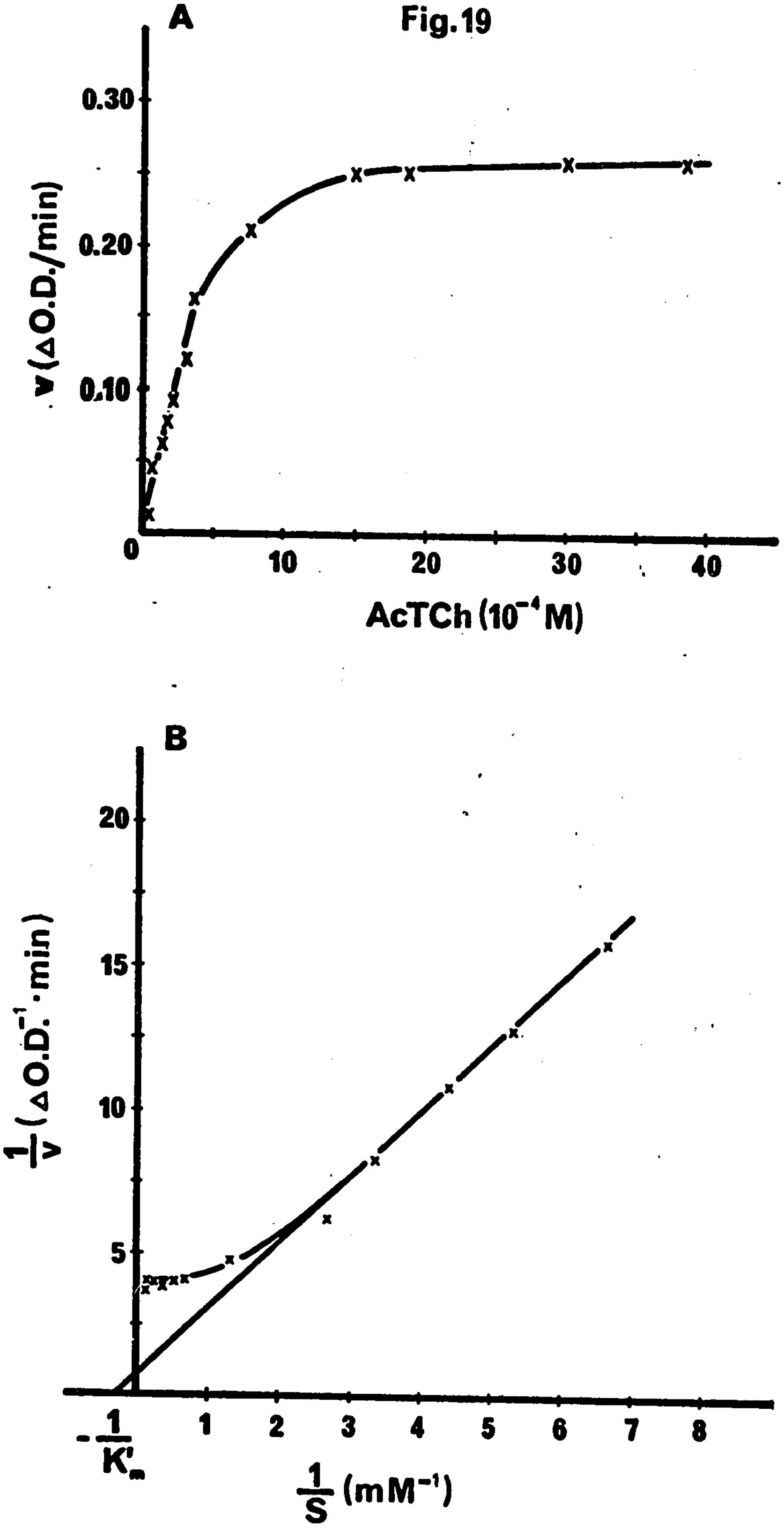
A. Effect of Substrate Concentration on the Enzymatic Hydrolysis of Acetylthiocholine Iodide

Bovine erythrocyte AChE (0.1 mg) (Sigma) was incubated at 37°C with different concentrations of AcTCh. The initial reaction velocity in change in O.D. (Δ O.D.) per minute was determined in duplicate at each concentration. The mean values from 5 experiments are presented. (TABLE XXVII).

B. Lineweaver-Burk Reciprocal Plot

The same data were recalculated according to Lineweaver and Burk. The apparent K'_m value was determined graphically to be 3×10^{-4}M AcTCh.

Fig.19


A
0.30
0.20
0.10
v (ΔO.D./min)
0
10
20
30
40
AcTCh (10⁻⁴ M)
B
20
15
10
5
1/v (ΔO.D.⁻¹ · min)
- 1/K'm
1
2
3
4
5
6
7
8
1/S (mM⁻¹)

by the addition of 0.1 ml of a 0.05% solution of pure bovine erythrocyte AChE made up in 0.1M sodium phosphate buffer, pH 7.4. After intervals of 2,4,6, and 8 minutes, the reaction was terminated by the addition of 0.1 ml 25% (w/v) TCA. Duplicate aliquots of 0.2 ml were withdrawn and the sulphydryl content determined as a change in optical density at 520 mµ according to Grunert and Phillips (480).

The data was calculated to give initial reaction velocities and presented as Lineweaver-Burk reciprocal plots (1/v versus 1/S) in order to establish the competitive or non-competitive nature of the inhibition and as Dixon plots (1/v versus I) in order to confirm graphically the apparent inhibitory constant, K_i'.

For all compounds examined, the inhibition was of a competitive type and the inhibitory constants were taken to be a measure of the affinity of any given compound for the enzyme. As the assay system for enzymatic activity depended upon the production of sulphydryl groups by hydrolysis, no measure of simultaneous cleavage of the inhibitor was made.

An examination of the apparent K_i' values, which were taken to be a measure of the affinities of the compounds for the active centre of AChE, proved of interest.

Eserine sulphate had a K_i' value of 2.9×10^{-8}M, in agreement with the literature that this is an extremely potent competitive inhibitor (496).

Figure 20

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine by Eserine Sulphate

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, eserine sulphate, were varied. The mean values of 4 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXVIII).

Eserine sulphate concentrations (10^{-7}M):

(a) 0.30
(b) 0.10
(c) 0

B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a) 0.30
(b) 0.45
(c) 0.75
(d) 1.50

The apparent K_i' value obtained graphically was 2.9×10^{-8}M.

Fig.20

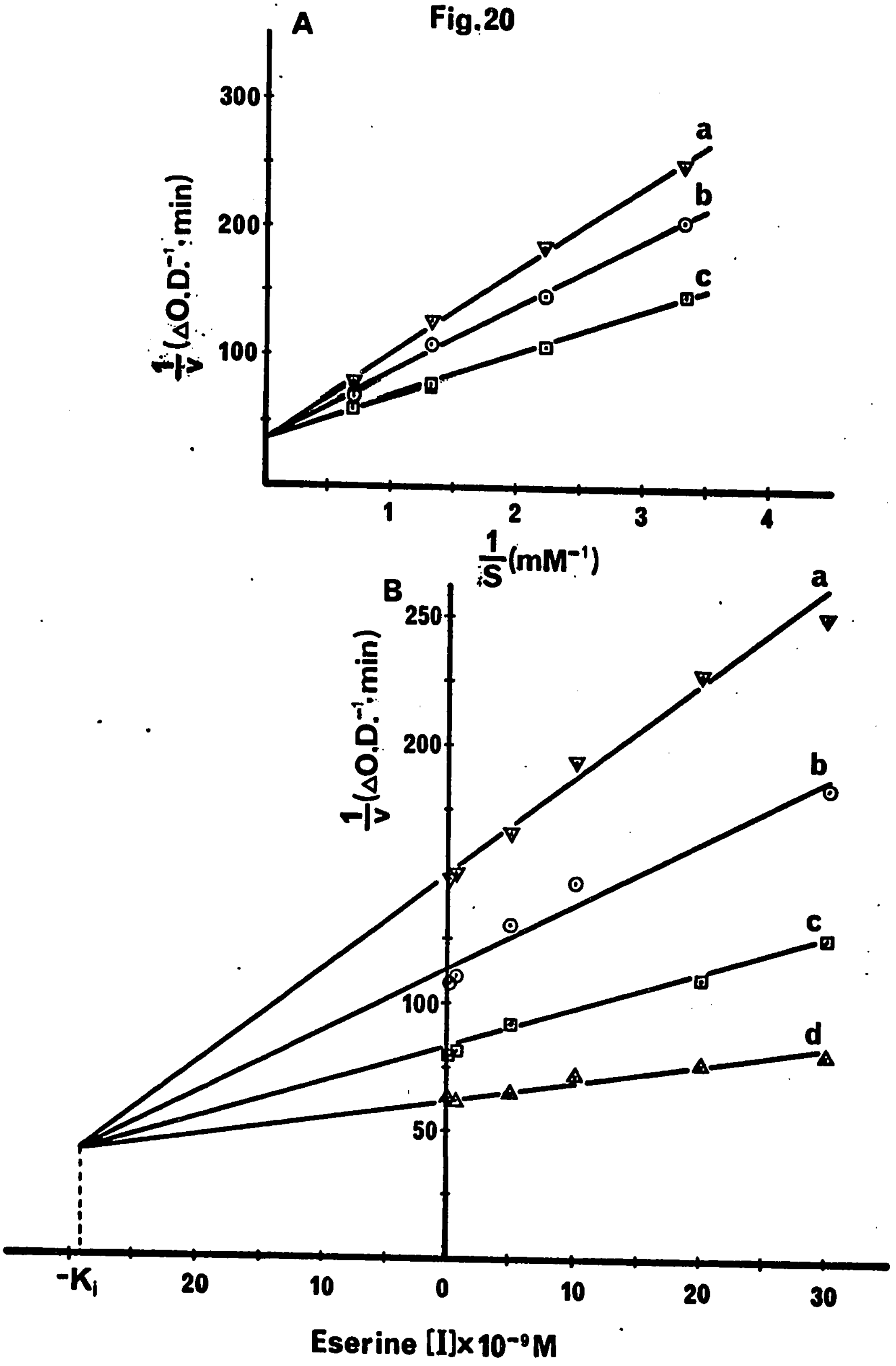

A
300
200
100
$\frac{1}{v}$(ΔO.D.$^{-1}$·min)
a
b
c
1
2
3
4
$\frac{1}{S}$(mM^{-1})
B
250
200
100
50
$\frac{1}{v}$(ΔO.D.$^{-1}$·min)
a
b
c
d
-K$_i$
20
10
0
10
20
30
Eserine [I]×10^{-9}M

Figure 21

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine by Acetylcholine Iodide

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, acetylcholine iodide (ACh), were varied. The mean values of 5 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXIX).

ACh concentrations (mM):

(a) 15.0
(b) 7.5
(c) 1.50
(d) 0.00

B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a) 1.50
(b) 3.25
(c) 7.5
(d) 15.0

The apparent K_i' value obtained graphically was 1.0×10^{-3}M.

Fig.21

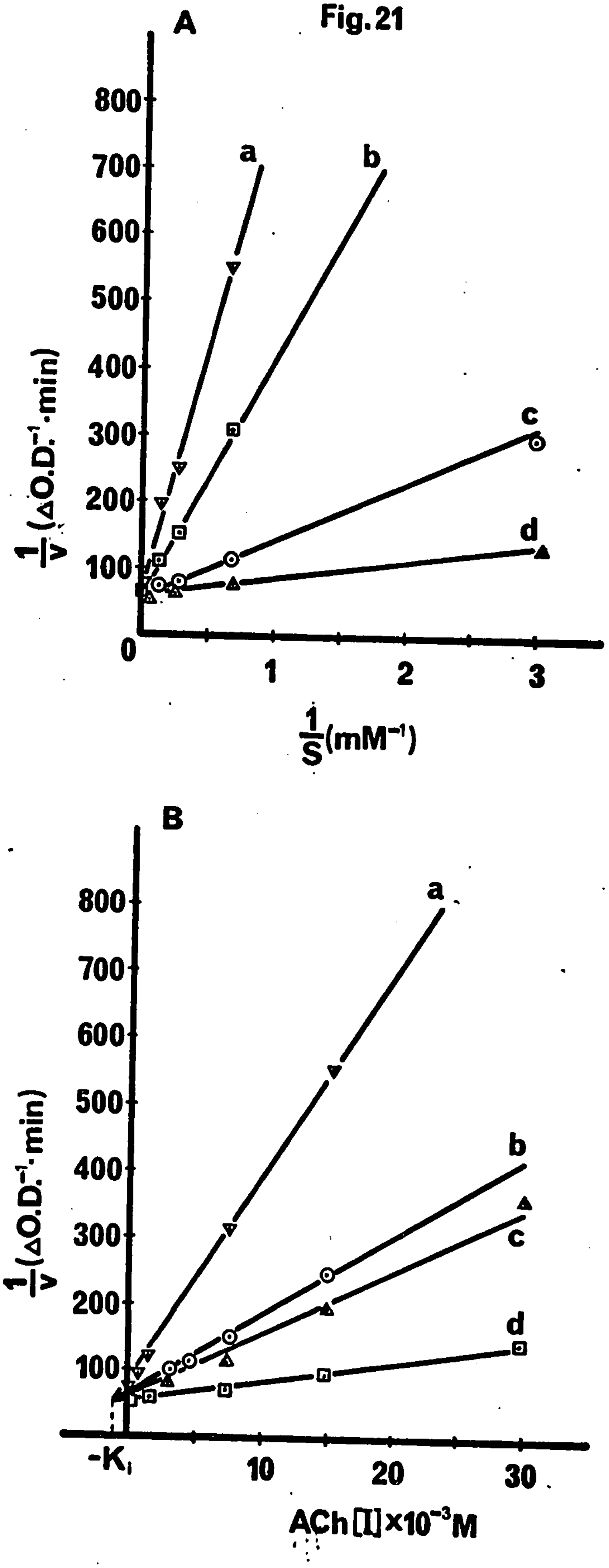

A
800
700
600
500
400
300
200
100
0
a
b
c
d
1
2
3
$\frac{1}{v}$(ΔO.D.$^{-1}$·min)
$\frac{1}{S}$(mM^{-1})
B
800
700
600
500
400
300
200
100
a
b
c
d
-K$_i$
10
20
30
$\frac{1}{v}$(ΔO.D.$^{-1}$·min)
ACh[I]×10^{-3}M

Figure 22

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Acetyl-ß-methylcholine Bromide

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, acetyl-ß-methylcholine bromide (Ac-ß-MeCh), were varied. The mean values of 3 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXX).

Ac-ß-MeCh concentrations (mM):

(a) 15.0
(b) 7.5
(c) 3.5
(d) 0

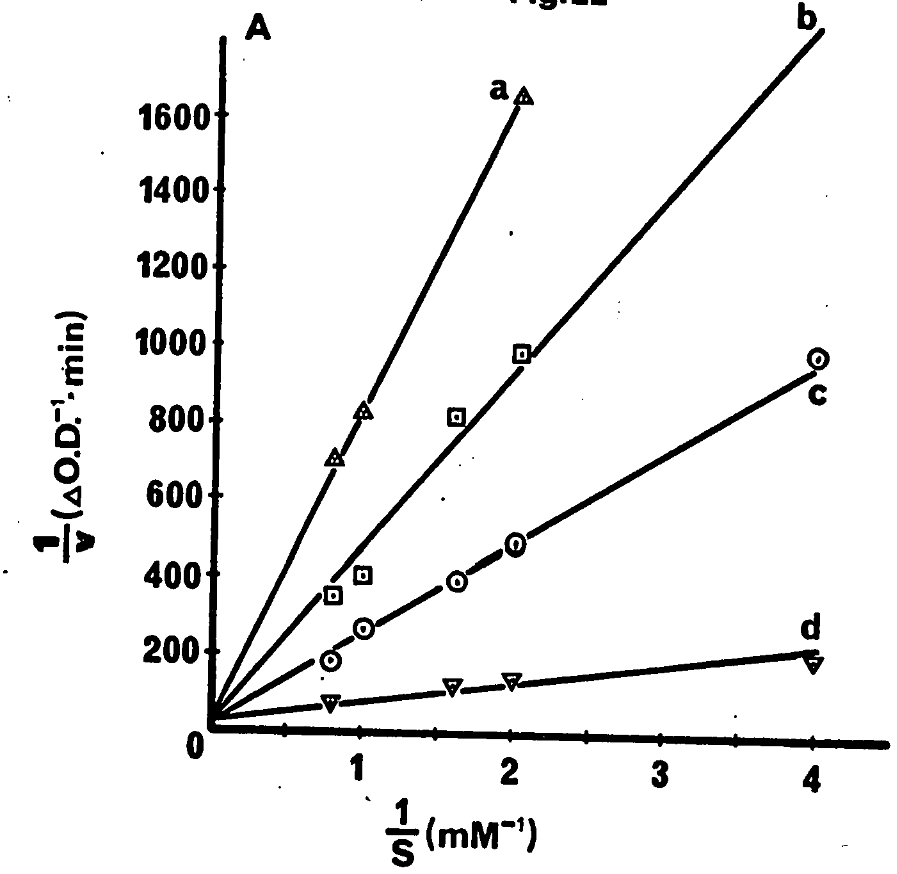
B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a) 0.50
(b) 0.625
(c) 1.00
(d) 1.25

The apparent K_i' value obtained graphically was 1.0×10^{-3}M.

Fig.22


A
a
b
c
d
1600
1400
1200
1000
800
600
400
200
0
1
2
3
4
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
$\frac{1}{S}$ (mM^{-1})

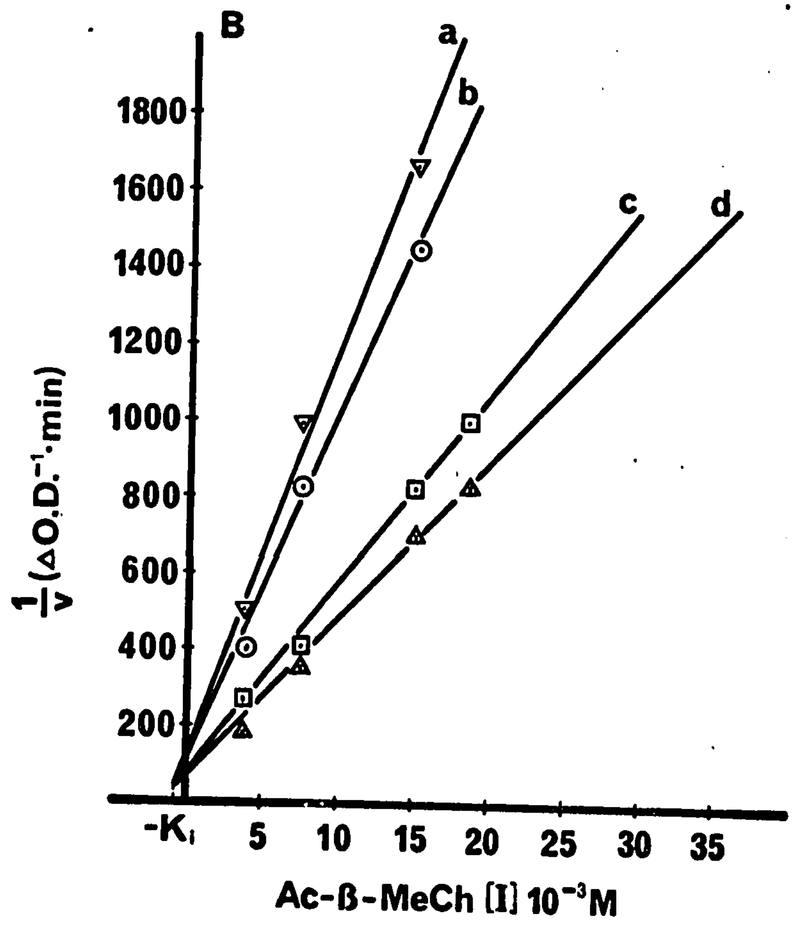

B
a
b
c
d
1800
1600
1400
1200
1000
800
600
400
200
-K$_i$
5
10
15
20
25
30
35
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
Ac-ß-MeCh [I] 10^{-3}M

Figure 23

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Propionylcholine Chloride

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte AChE (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, propionylcholine (PCh), were varied. The mean values of 4 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXXI).

PCh concentrations (mM):

(a)	10.0	(d)	2.50
(b)	5.0	(e)	1.25
(c)	3.75	(f)	0

B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a)	0.375	(c)	1.50
(b)	0.750	(d)	3.00

The apparent K_i' value obtained graphically was 1.06×10^{-3}M.

Fig,23

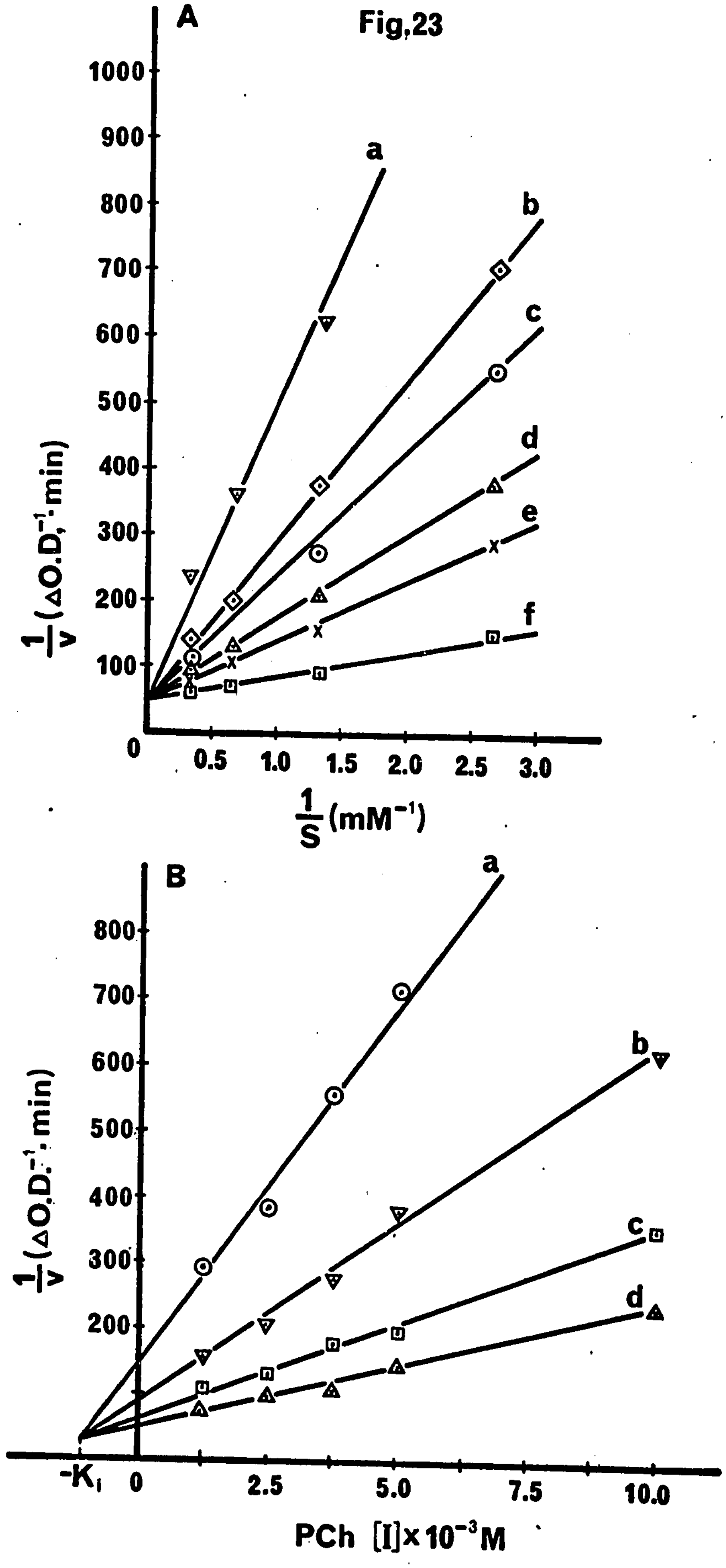

A
1000
900
800
700
600
500
400
300
200
100
0
0.5
1.0
1.5
2.0
2.5
3.0
a
b
c
d
e
f
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
$\frac{1}{S}$ (mM^{-1})
B
800
700
600
500
400
300
200
a
b
c
d
-K$_i$
0
2.5
5.0
7.5
10.0
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
PCh [I]×10^{-3}M

Figure 24

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by dl-Acetylcarnitylcholine Chloride

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, dl-acetylcarnitylcholine (dl-ACCh), were varied. The mean values of 5 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXXII).

dl-ACCh concentrations (mM):

(a) 10.0
(b) 6.0
(c) 2.5
(d) 0.75

B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a) 0.33
(b) 0.58
(c) 1.50
(d) 3.00

The apparent K_i' value obtained graphically was 0.8×10^{-3}M.

Fig.24

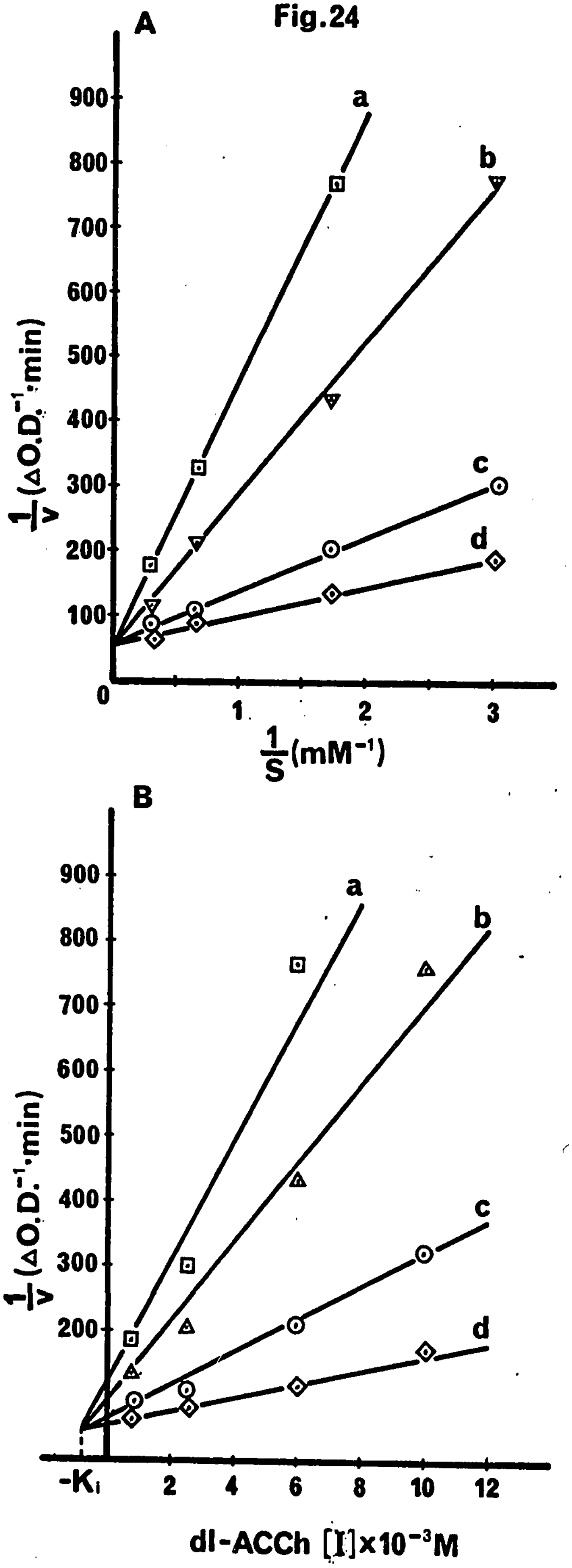

A
900
800
700
600
500
400
300
200
100
0
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
a
b
c
d
1
2
3
$\frac{1}{S}$ (mM^{-1})
B
900
800
700
600
500
400
300
200
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
a
b
c
d
-K$_i$
2
4
6
8
10
12
dl-ACCh [I]×10^{-3}M

Figure 25

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Succinyldicholine Chloride

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, succinyldicholine (SDCh), were varied. The mean values of 3 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXXIII).

SDCh concentrations (mM):

(a) 4.0
(b) 2.0
(c) 0.5
(d) 0

B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a) 0.375
(b) 0.75
(c) 2.25

The apparent K_i' value obtained graphically was 2.0×10^{-3}M.

Fig.25

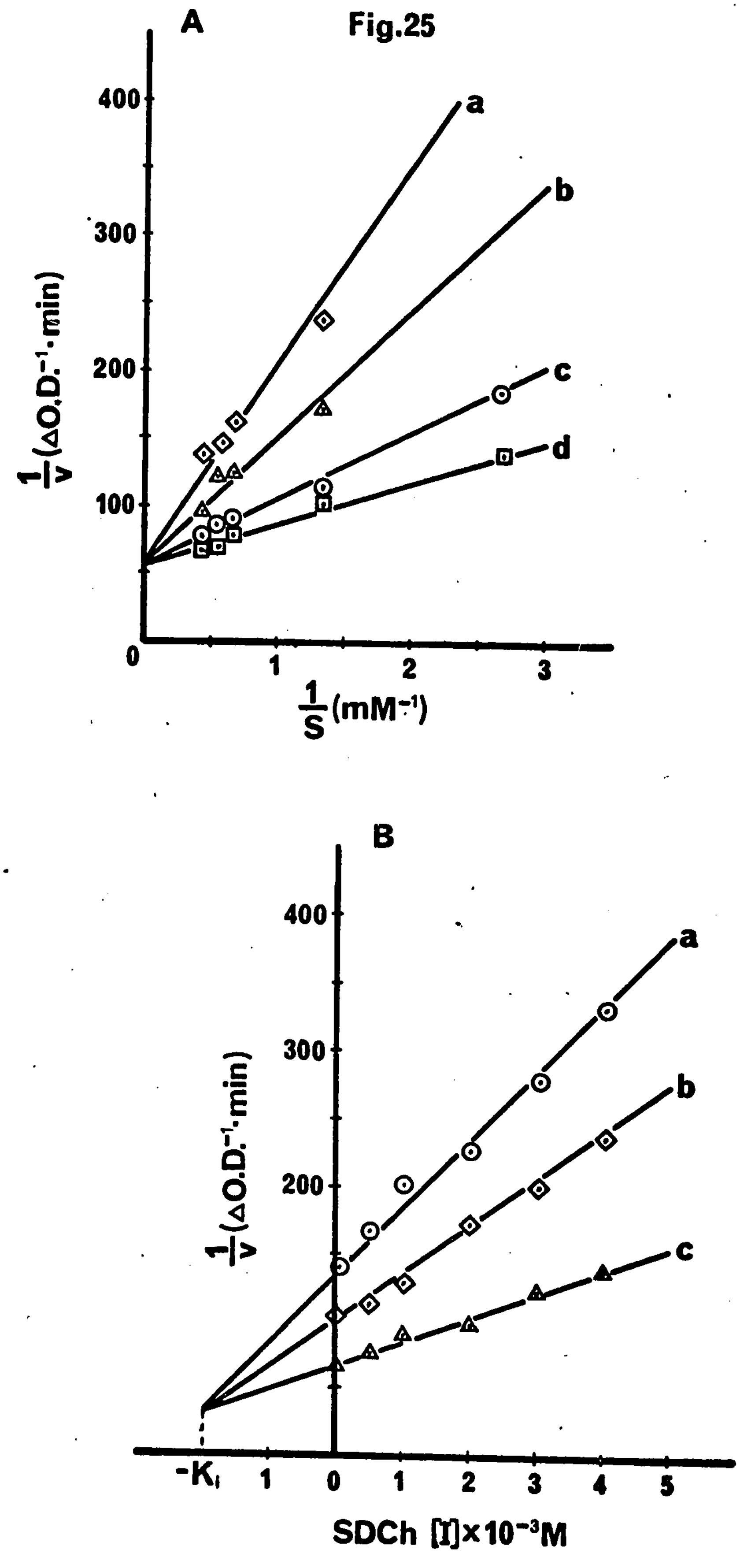

A
400
300
200
100
0
1
2
3
$\frac{1}{v}$(ΔO.D.$^{-1}$·min)
$\frac{1}{S}$(mM^{-1})
a
b
c
d
B
400
300
200
100
$\frac{1}{v}$(ΔO.D.$^{-1}$·min)
-K$_i$
1
0
1
2
3
4
5
SDCh [I]×10^{-3}M
a
b
c

Figure 26

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by l-Carnitylcholine Chloride

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, l-carnitylcholine (l-CCh), were varied. The mean values from 3 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXXIV).

l-CCh concentrations (mM):

(a) 9.36
(b) 7.02
(c) 4.68
(d) 2.34

B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a) 1.0
(b) 1.5
(c) 2.5
(d) 3.0
(e) 10.0

The apparent K_i' value obtained graphically was 6.0×10^{-3}M.

Fig. 26

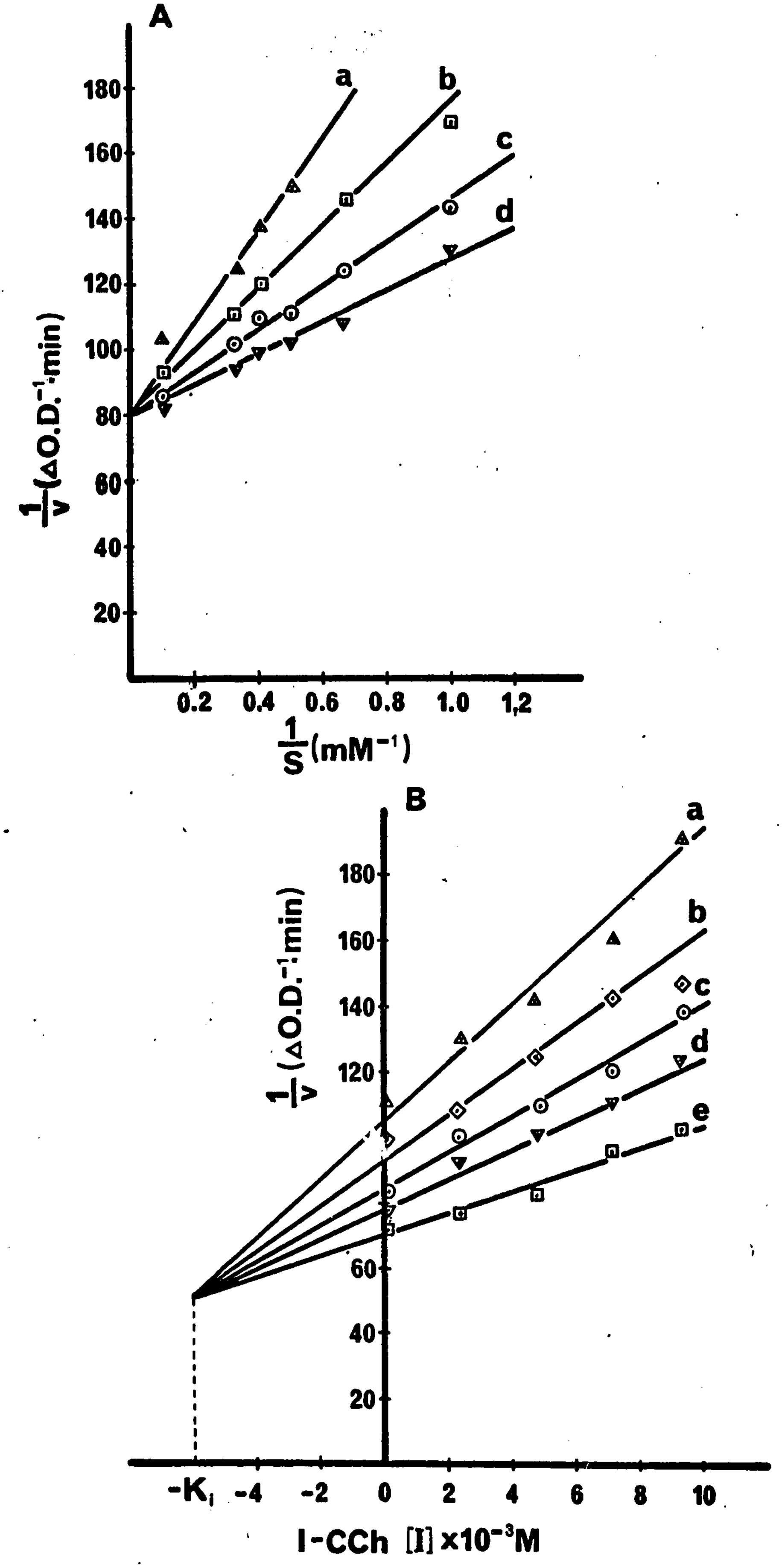

A
a
b
c
d
180
160
140
120
100
80
60
40
20
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
0.2
0.4
0.6
0.8
1.0
1,2
$\frac{1}{S}$ (mM^{-1})
B
a
b
c
d
e
180
160
140
120
60
40
20
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
-K$_i$
-4
-2
0
2
4
6
8
10
I-CCh [I] ×10^{-3}M

Figure 27

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by d-Carnitylcholine Iodide

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (AChE). The concentrations of both the substrate, AcTCh, and the inhibitor, d-carnitylcholine chloride (d-CCh), were varied. The mean values of 3 experiments are presented as Lineweaver-Burk reciprocal plots (TABLE XXXV).

d-CCh concentrations (mM):

(a) 0.1
(b) 0.2
(c) 0.3
(d) 0.4

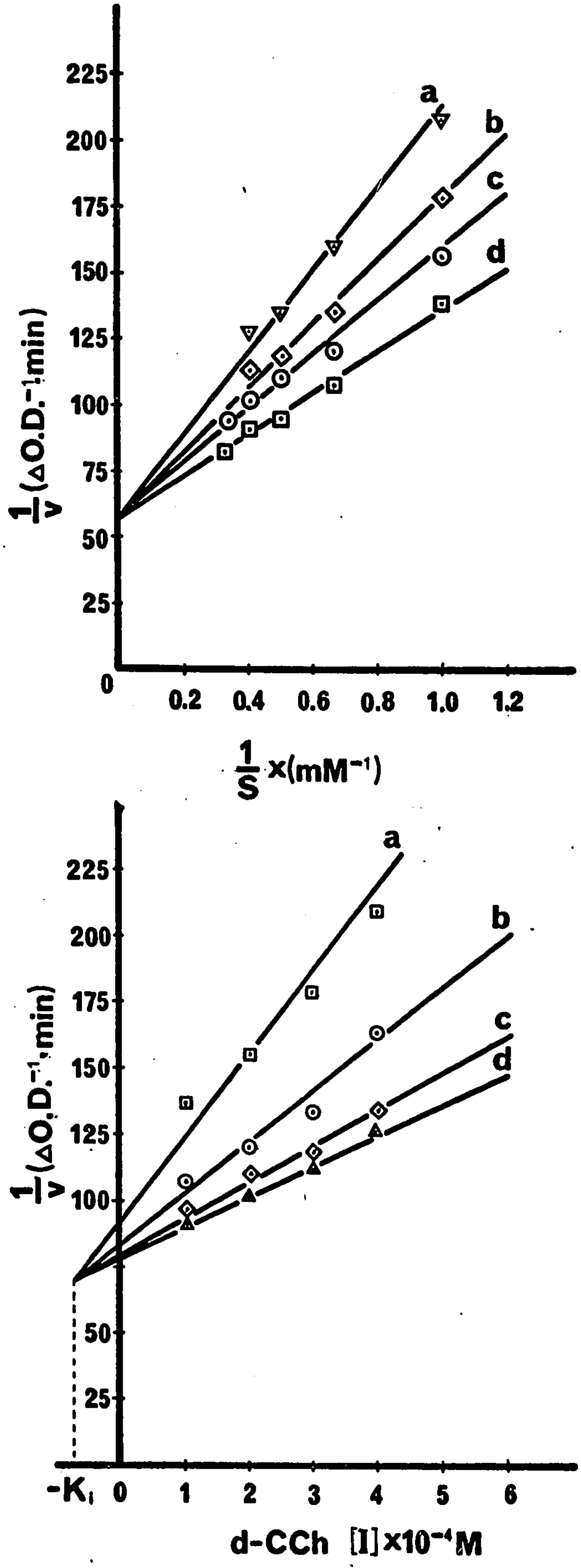
B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a) 1.0
(b) 1.5
(c) 2.0
(d) 2.5

The apparent K_i' value obtained graphically was 6.5×10^{-3}M.

Fig.27



$\frac{1}{v}$(ΔO.D.$^{-1}$.min)
225
200
175
150
125
100
75
50
25
0
0.2
0.4
0.6
0.8
1.0
1.2
$\frac{1}{S}$ x(mM^{-1})
a
b
c
d
$\frac{1}{v}$(ΔO.D.$^{-1}$.min)
225
200
175
150
125
100
50
25
-K$_i$
0
1
2
3
4
5
6
d-CCh [I]×10^{-4}M
a
b
c
d

Figure 28

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Butyrylcholine Iodide

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, butyrylcholine iodide (BuCh), were varied. The mean values of 4 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXXVI).

BuCh concentrations (mM):

(a) 30.0
(b) 15.0
(c) 7.5
(d) 0

B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a) 0.187
(b) 0.375
(c) 0.75
(d) 0.938
(e) 1.50

The apparent K_i' value obtained graphically was 9.0×10^{-3}M.

Fig.28

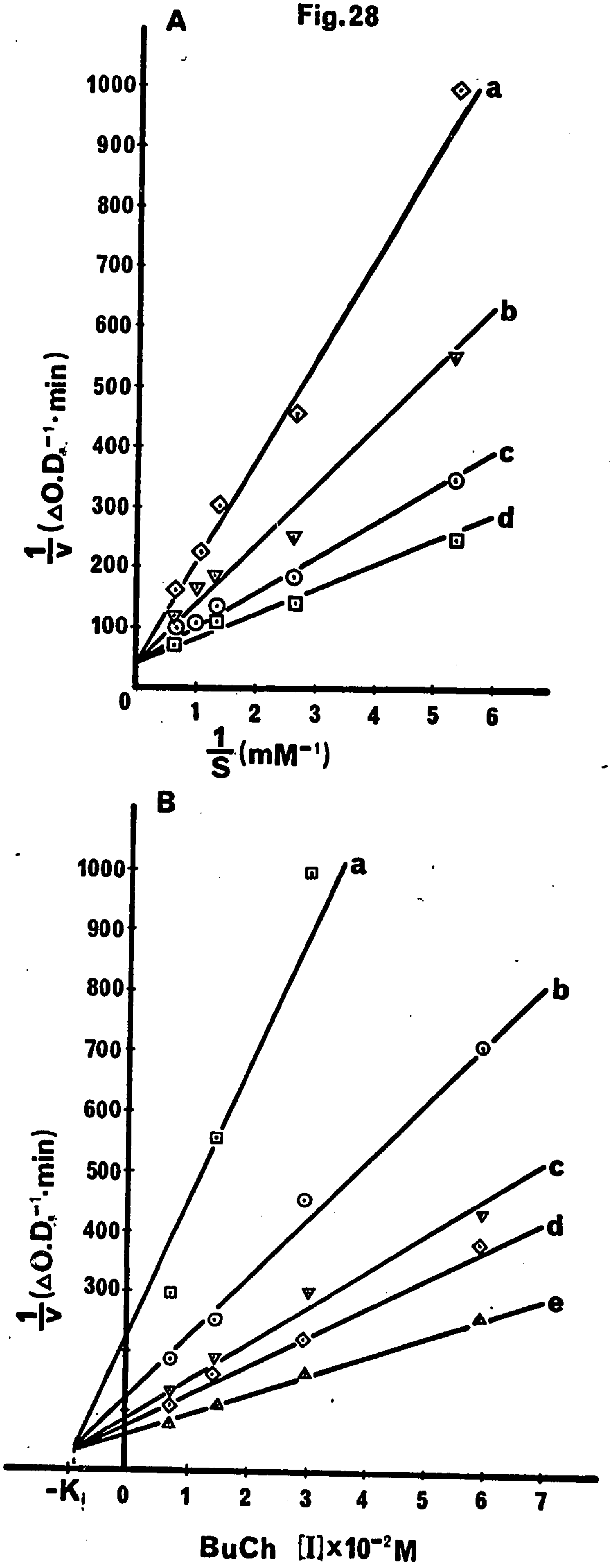

A
1000
900
800
700
600
500
400
300
200
100
0
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
a
b
c
d
1
2
3
4
5
6
$\frac{1}{S}$ (mM^{-1})
B
1000
900
800
700
600
500
400
300
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
a
b
c
d
e
$-K_i$
0
1
2
3
4
5
6
7
BuCh [I]×10^{-2}M

Figure 29

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Choline Chloride

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, choline chloride, were varied. The mean values of 4 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXXVII).

Choline concentrations (10^{-2}M):

(a) 60
(b) 40
(c) 20
(d) 0

B. The same data were recalculated according to Dixon.

(a) 1.0
(b) 1.5
(c) 2.0
(d) 3.0
(e) 15.0

The apparent K_i' value obtained graphically was 10×10^{-2}M.

Fig.29

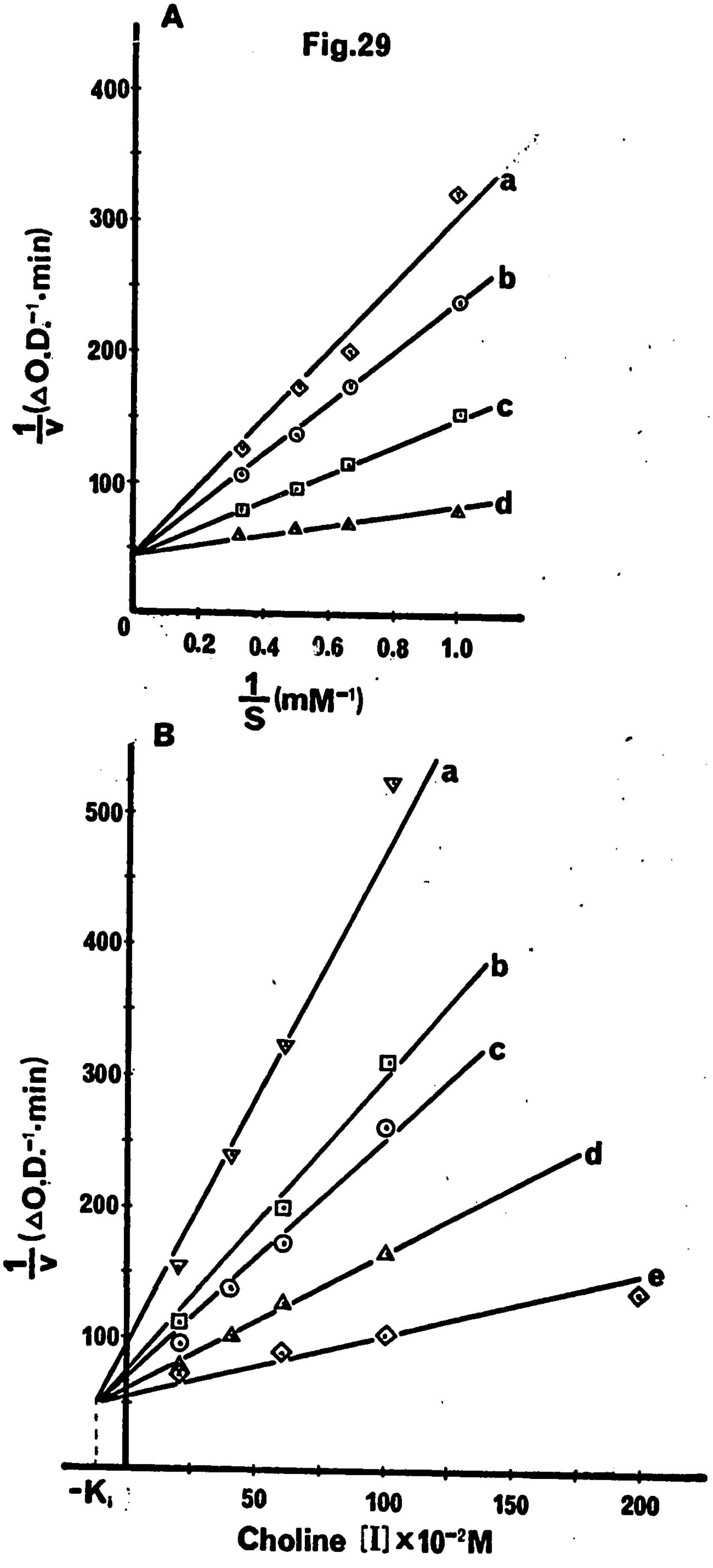

A
400
300
200
100
0
0.2
0.4
0.6
0.8
1.0
$\frac{1}{v}$(ΔO.D.⁻¹·min)
$\frac{1}{S}$ (mM⁻¹)
a
b
c
d
B
500
400
300
200
100
-Kᵢ
50
100
150
200
$\frac{1}{v}$ (ΔO.D.⁻¹·min)
Choline [I] x10⁻²M
a
b
c
d
e

Figure 30

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by dl-Acetylcarnitine Chloride

A. Incubation was carried out at 37°C in the presence of 0.05 mg bovine erythrocyte acetylcholinesterase (Sigma). The concentrations of both the substrate, AcTCh, and the inhibitor, dl-acetylcarnitine (dl-AC), were varied. The mean values of 4 experiments are presented as Lineweaver-Burk reciprocal plots. (TABLE XXXVIII).

dl-AC concentrations (mM):

(a) 125
(b) 50
(c) 25
(d) 0

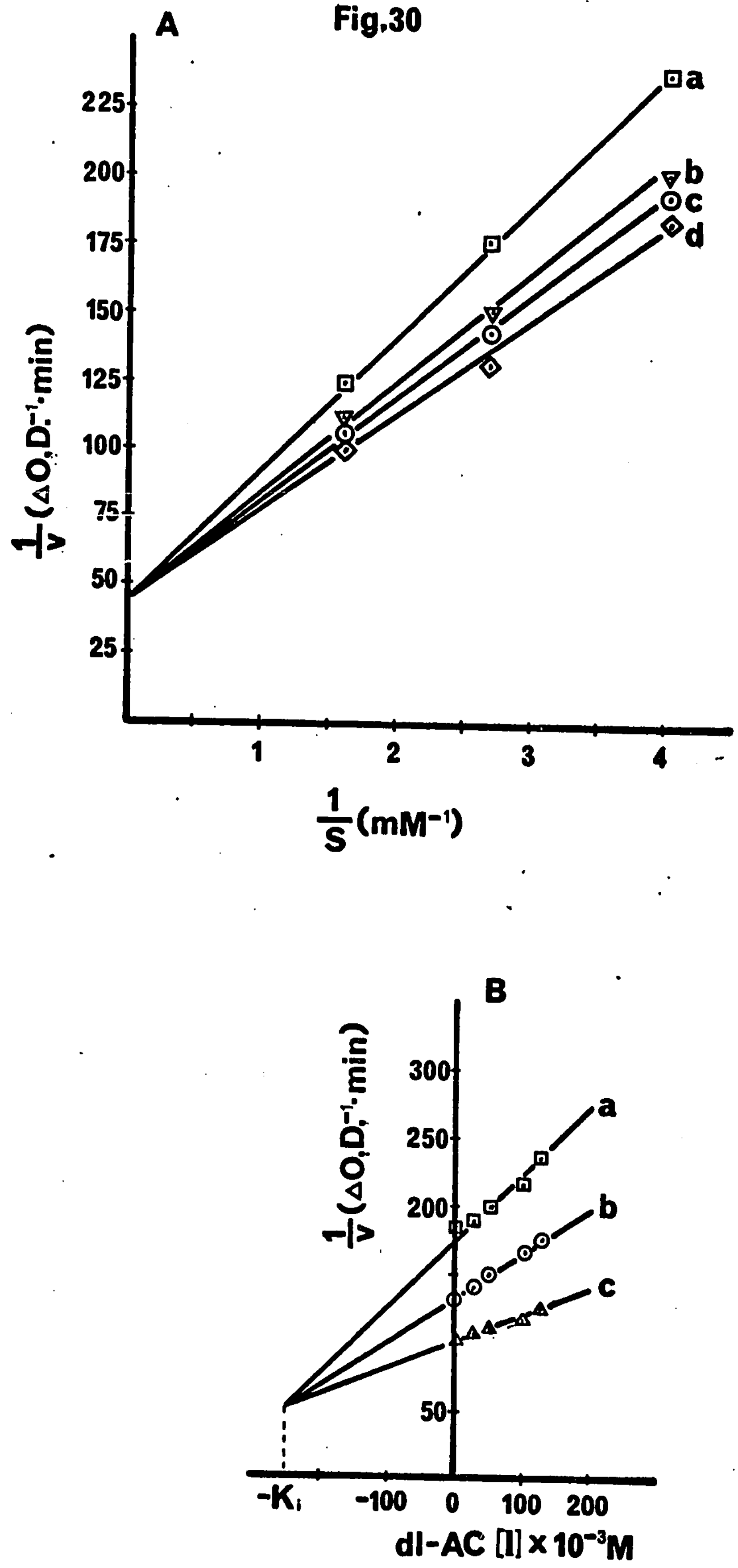
B. The same data were recalculated according to Dixon.

AcTCh concentrations (mM):

(a) 0.25
(b) 0.375
(c) 0.625

The apparent K_i' value obtained graphically was 25 x 10^{-2}M.

Fig.30


A
225
200
175
150
125
100
75
50
25
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
1
2
3
4
$\frac{1}{S}$ (mM^{-1})
a
b
c
d
B
300
250
200
50
$\frac{1}{v}$ (ΔO.D.$^{-1}$·min)
a
b
c
-K$_i$
-100
0
100
200
dl-AC [I] × 10^{-3}M

ACh, the proposed true substrate of the enzyme, had the same K_i' ($1.0 \times 10^{-3}M$) as did Ac-ß-MeCh, and PCh had a very similar value ($1.06 \times 10^{-3}M$). This was in accord with the finding that all three compounds are excellent substrates for AChE. Of particular interest, in this respect, therefore was the low K_i' value for dl-ACCh ($0.8 \times 10^{-3}M$). This would appear to indicate that even a racemic mixture of this compound had a marked affinity for the enzyme active centre. It also posed the problem of which isomer was the more powerful inhibitor, a problem which remains to be studied, as the purified isomers are not available for investigation.

BuCh, a poor substrate for AChE, had a K_i' value which was 9 times greater than that for ACh. Intermediate between these were the linear bis-quaternary compounds, SDCh and CCh, which were moderately good inhibitors, while not substrates, of the enzyme.

Choline, in agreement with previous findings from the literature, was a poor inhibitor of AChE, as would be expected in view of the function of the enzyme.

Particular attention was also paid to dl-AC with a very high K_i' value of $25 \times 10^{-2}M$. This compound was a weaker inhibitor of the enzyme than even choline, a fact which would appear to be unusual in view of its structure, which closely resembles that of ACh. This lack of inhibition would appear to be supporting evidence for the existence of this compound as a ring structure.

Section G. Nature of the Products of the Hydrolysis of dl-Acetylcarnitylcholine Chloride

Enzymatic hydrolysis of dl-ACCh by acetylcholinesterase (AChE) led to a maximum decrease of only 25% of the ester bonds in the reaction mixture. It was concluded, therefore, that hydrolysis brought about destruction of only one ester bond of the four present in the two stereoisomers; the enzyme being known to be stereospecific for other substrates (380,383).

It was assumed on the basis that the naturally occurring form of carnitine is the l-isomer, that the isomer hydrolyzed by the enzyme was probably the l-ACCh. On the basis of biological activity studies in pharmacological test systems (471,473), this assumption appeared to be correct.

The enzymatic destruction of l-ACCh might therefore give rise to one of the following pairs of reaction products:

$$\text{l-ACCh} \xrightarrow{\text{AChE}} \text{acetate} + \text{carnitylcholine (CCh)} \qquad \text{(i)}$$

$$\text{l-ACCh} \xrightarrow{\text{AChE}} \text{l-acetylcarnitine (AC)} + \text{choline} \qquad \text{(ii)}$$

1. Determination of "Free Choline"

In order to detect the possible presence of the end-product choline in the hydrolytic incubation mixtures, the micromethod of Appleton for "free" choline was tried. The method entailed determining the optical density of an iodine complex of the compound at 365 mμ. When the other compounds possibly occurring in the incubation mixture were tested for control purposes, it was found that AC, CCh, ACCh and even ACh gave a high optical density at 295 and 365 mμ. Identical spectra on the Beckman DK spectrophotometer, exhibiting peaks at 295 and 365 mμ, were obtained with a variety of compounds chosen at random, the majority of which contained a quaternary nitrogen group. Positive results were also obtained with the following compounds: neostigmine, physostigmine, betaine, acetyl-ß-methylcholine, butyrylcholine, propionylcholine, ephedrine hydrochloride, atropine sulphate, l-methionine, and a serotonin-creatinine complex. Compounds not reacting with the iodine reagent included l-arginine hydrochloride, l-histidine, l-carnosine, sodium barbitone, l-asparagine, l-proline, and dl-lysine. The method therefore could not be employed to detect choline in the incubation hydrolytic mixture.

2. Enzymatic Determination of Choline

The detection of choline in the hydrolytic incubation mixtures was next investigated with the choline oxidase system.

Choline is metabolized *in vivo* to glycine betaine by the activities of two enzymes, choline dehydrogenase and betaine aldehyde dehydrogenase, and the choline oxidase system can be considered to involve both enzymes. A preparation of soluble choline dehydrogenase was prepared by extraction of acetone-ether dried rat liver mitochondria with sodium choleate (Methods, Part D,). A spectrophotometric assay involved determining the optical density changes at 607 mμ of the oxidizable blue dye 2,6-dichlorophenolindophenol.

The standard assay method for choline was studied first using 0.2 ml of a 2% choline chloride solution. The optical density of the mixture containing choline plus enzyme decreased rapidly but did not reach a constant value. The control mixtures, containing no choline, also gave reduction of the dye due to the presence of endogenous metabolites. The rate of decrease of O.D. was, in every case, greater for the mixture containing choline (Figure 31).

Subtraction of the change in O.D. per unit time of the control mixture from that of the experimental mixture gave a straight line for 4 minutes at 2 mg choline chloride (Figure 31,B,C) from which the initial velocity could be determined.

The effect of varying substrate concentration was examined (Figure 32) and the initial velocity was found to vary with choline concentration in a linear manner to 1.2 mg choline and to reach a maximum at 2 mg choline chloride.

The ability of the enzyme to oxidize other related compounds was examined. Figure 32, B,C showed that dl-AC was unaffected by choline dehydrogenase at a concentration of 1 mg. Other compounds which

Figure 31

Spectrophotometric Assay for Choline Dehydrogenase

A. Standard Assay Results

The assay was carried out according to Williams (486,487). The incubation mixtures contained: 1.0 ml Krebs-Ringer phosphate buffer, pH 6.8, 0.2 ml of 2.0% choline chloride or distilled water, 0.5 ml 10 mg% 2,6-dichlorophenolindophenol, and distilled water to a total volume of 2.2 ml. Optical density (O.D.) readings at 607 mμ were taken at 30 second intervals after the addition of 1.0 ml mitochondrial suspension. (TABLE XXXIX).

(a) Choline concentration - 2.0 mg
(b) Choline concentration - 0

B. Determination of Change of Optical Densities with Time

(a) Choline concentration - 0
(b) Choline concentration - 2.0 mg

C. Determination of Initial Velocity

$\Delta O.D._{\text{Exptl.}} - \Delta O.D._{\text{Control}}$ versus time

Fig.31

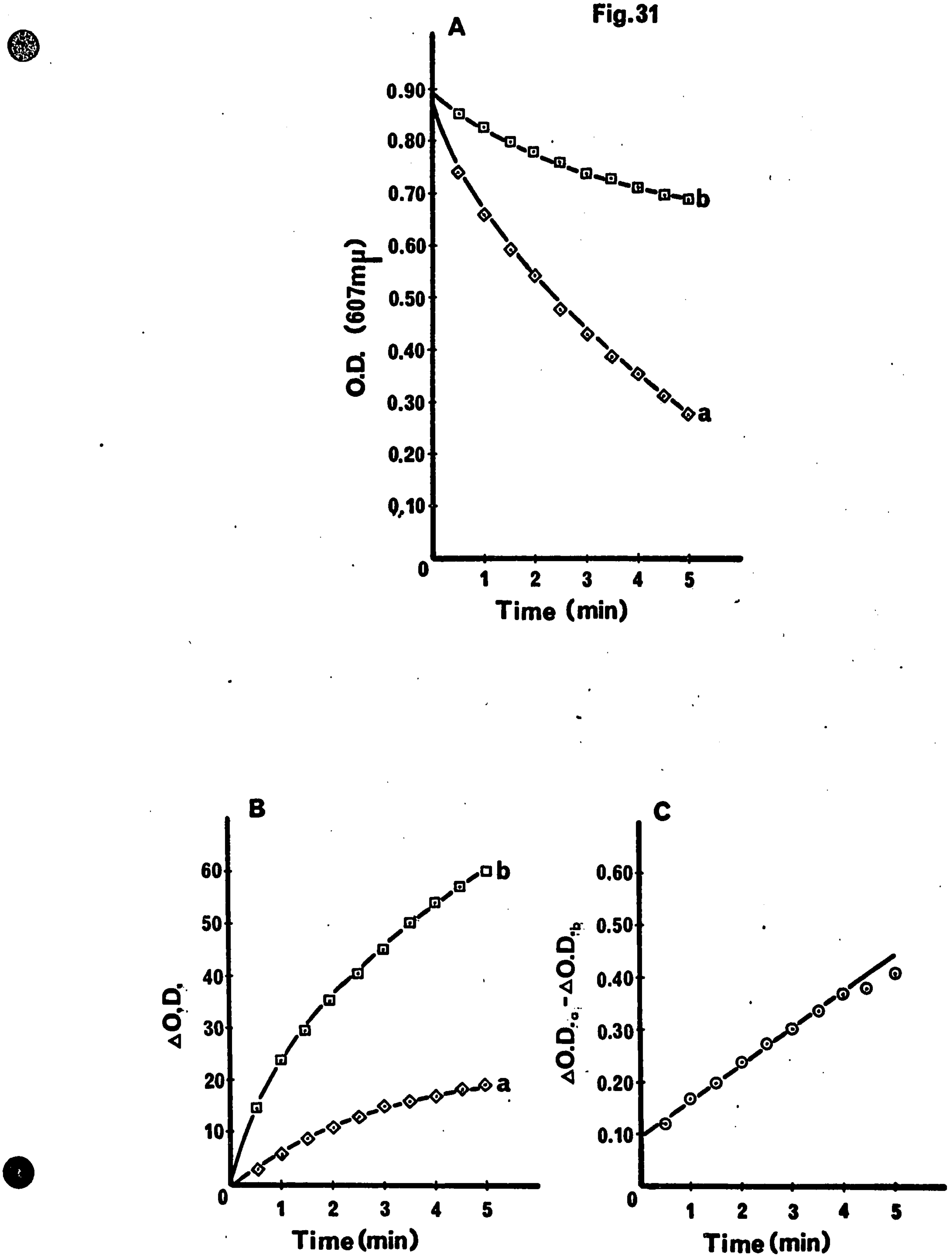

A
O.D. (607mμ)
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0,10
0
1
2
3
4
5
Time (min)
a
b
B
ΔO.D.
60
50
40
30
20
10
0
1
2
3
4
5
Time (min)
b
a
C
ΔO.D.a - ΔO.D.b
0.60
0.50
0.40
0.30
0.20
0.10
0
1
2
3
4
5
Time (min)

Figure 32

Activity of Choline Dehydrogenase

A. Effect of Substrate Concentration on Enzyme Activity

The initial velocity of the reaction in $\Delta O.D._{Exptl.} - \Delta O.D._{Control}$ per minute is recorded with changes of choline concentration. The results with 2 different enzyme preparations are presented. (TABLE XL).

B. Standard Assay with Choline and dl-Acetylcarnitine as Substrates

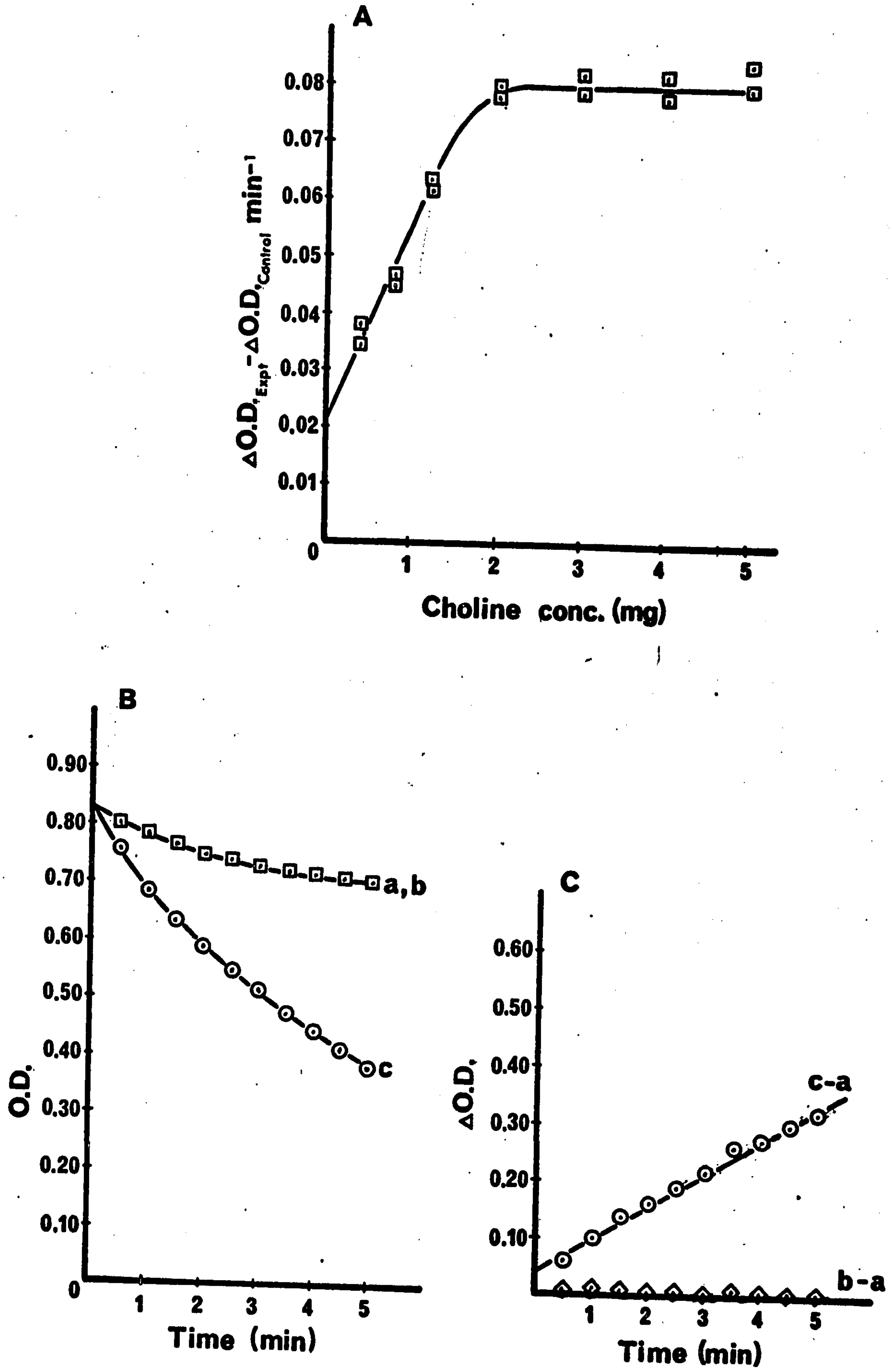
The standard assay was carried out according to Williams (487,488). The incubation mixtures contained: 1.0 ml Krebs-Ringer phosphate buffer, pH 6.8, 0.2 ml substrate as specified below, 0.5 ml 10 mg% 2,6-dichlorophenolindophenol, and distilled water to a total volume of 2.2 ml. Optical density readings following the addition of 1.0 ml enzyme suspension were recorded at 607 mμ. (TABLE XLI).

(a) Control - no substrate
(b) dl-Acetylcarnitine - 2.0 mg and/or
(b) dl-Acetylcarnitine - 2 mg + choline - 1 mg
(c) Choline - 1 mg

C. Enzyme Activity

Enzymatic activity calculated as changes in O.D. with time.

Fig.32


A
ΔO.D.$_{Expt}$ – ΔO.D.$_{Control}$ min^{-1}
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1
2
3
4
5
Choline conc. (mg)
B
O.D.
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
1
2
3
4
5
a, b
c
Time (min)
C
ΔO.D.
0.60
0.50
0.40
0.30
0.20
0.10
1
2
3
4
5
c-a
b-a
Time (min)

were unaffected by choline dehydrogenase included dl-CCh, l-CCh, dl-ACCh and dl-carnitine.

It was found, however, that the action of choline dehydrogenase on 1 mg choline chloride was completely inhibited by 1 mg dl-AC, shown in Figure 32, B,C. Similar results were found with dl-CCh, dl-ACCh, and dl-carnitine. The presence of equal concentrations of choline and any of these compounds caused disappearance of enzymic activity in this system. This made the use of choline dehydrogenase for the identification of choline in a mixture of quaternary ammonium compounds impossible.

3. Ion-Exchange Column Chromatography

The method used to separate choline esters in rat brains by Gardiner and Whittaker (55) was studied as a possible method of identifying end products of enzymatic hydrolysis.

Sulphonic acid resins caused rapid destruction of esters as had been previously demonstrated (55).

The Amberlite XE-97 resin used by Gardiner and Whittaker and Dowex 50W-x4 were unsuitable for identifying the end products of dl-ACCh hydrolysis as it was found that neither pure AC nor pure ACCh could be eluted from the column at pH 4. Addition of strong acid destroyed the esters and therefore the method was abandoned for this purpose. The method was subsequently investigated more thoroughly by E. Vine of our laboratory (474).

4. Determination of Cholinester Hydrolase Activity on the Frog Rectus and Guinea Pig Ileum Preparations

The results of a typical bioassay for residual ACh-like activity on the guinea pig ileum preparation carried out in collaboration with A. Kato of our laboratory are shown in Figure 33,A. The pure compounds, l-ACCh, l-CCh, and ACh chloride were assayed and then, to the stock solutions made up in Tyrode's solution, approximately 10 μg purified bovine erythrocyte AChE (Sigma) was added. After incubation at 37°C for 5 minutes, suitable aliquots were assayed. This quantity of purified enzyme in Tyrode's solution was used as a control and was found to be inactive on the preparation. Samples of ACh and l-ACCh were found to be inactive after hydrolysis with AChE. This was expected if the end products of l-ACCh hydrolysis were the inactive l-AC and choline.

The activity of l-CCh was unchanged by incubation for up to 30 minutes with AChE, and ChE (purified horse serum cholinesterase) had no effect on l-ACCh and l-CCh although it completely inactivated ACh.

Similar experiments were carried out on the frog rectus preparation in collaboration with A. Kato of our laboratory. Typical results are presented in Figure 33,B. AChE action completely destroyed the nicotinic activity of both ACh and l-ACCh whereas the enzyme had no effect on l-CCh.

5. Paper Chromatography

The problem of the nature of the reaction products was also studied *in vitro* according to the method of Whittaker and Wijesundera (110) by paper chromatographic separation of the constituents of the reaction mixture at different stages of the hydrolysis.

Paper chromatography was carried out as described by Whittaker and Wijesundera (109), on Whatman No.4 filter paper. Immersion in iodine vapour was the method of detection of all possible reaction components except acetic acid.

Several solvent mixtures were examined (*n*-butanol: acetic acid: water 120:30:50; *n*-butanol:water saturated; *n*-propanol: benzyl alcohol: water 5:2:2; ethanol:water 8:1; *n*-propanol:formic acid:water 8:1:1; *n*-butanol: *n*-propanol:water 4:2:1) but acid and alkaline solvents were avoided due to the risk of hydrolysis on the paper. The solvent mixture chosen for the experiments was *n*-butanol:*n*-propanol:H_2O (4:2:1) as this gave the best separation of the reaction components.

The hydrolysis was determined colorimetrically at the same time using the modified Hestrin method (118). Results of the hydrolysis of dl-ACCh by bovine erythrocyte AChE (Sigma) as a function of time are shown in Table 11. The reaction was almost completed at 10 minutes and finished after 15 minutes incubation.

For chromatographic analysis of the reaction mixture at

Figure 33

A. "Muscarinic" Activity on the Guinea Pig Ileum Preparation

The muscarinic activity of ACh, l-ACCh and l-CCh were assayed on the guinea pig ileum preparation in a bath volume of 5 ml. Incubation medium: Tyrode's solution oxygenated with 95% O_2 - 5% CO_2 at 37°C. The responses elicited by the drugs in mμg or μg doses are indicated for each contraction. E represents samples of the drugs preincubated at 37°C for 10 minutes with approximately 10 μg purified bovine erythrocyte AChE before injection into the bath.

B. "Nicotinic" Activity on the Eserine-Sensitized Frog Rectus Abdominis Muscle Preparation

The nicotinic activity of ACh, l-ACCh, and l-CCh were assayed on the eserine-sensitized frog rectus preparation in a bath volume of 3 ml. Incubation medium: frog Locke's solution aerated with O_2 at room temperature. The responses produced by ACh and l-ACCh in mμg doses are indicated for each contraction. E represents samples of the drugs preincubated at 37°C for 5 minutes with approximately 10 μg purified bovine erythrocyte AChE (Sigma) before injection into the bath.

Fig.33

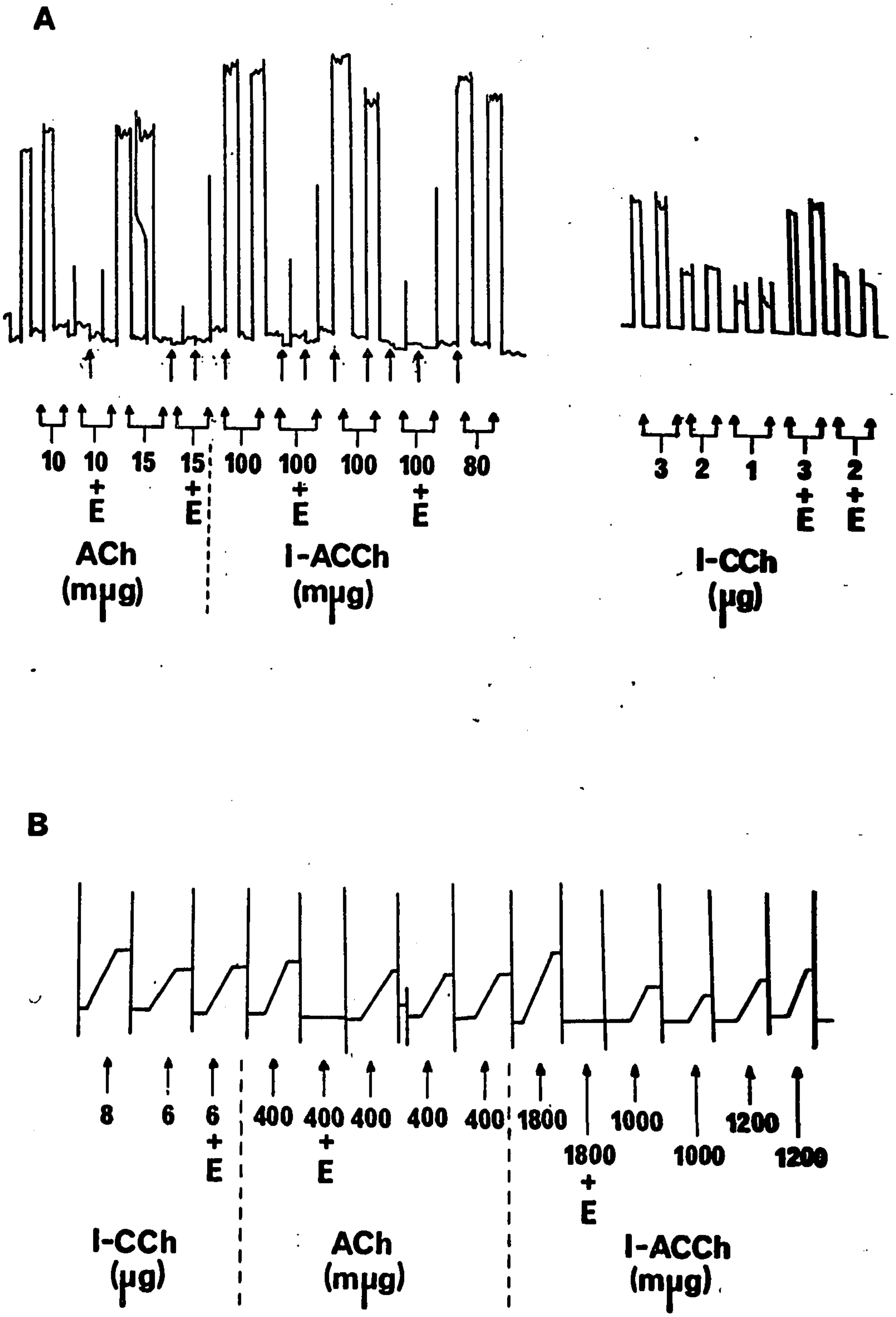
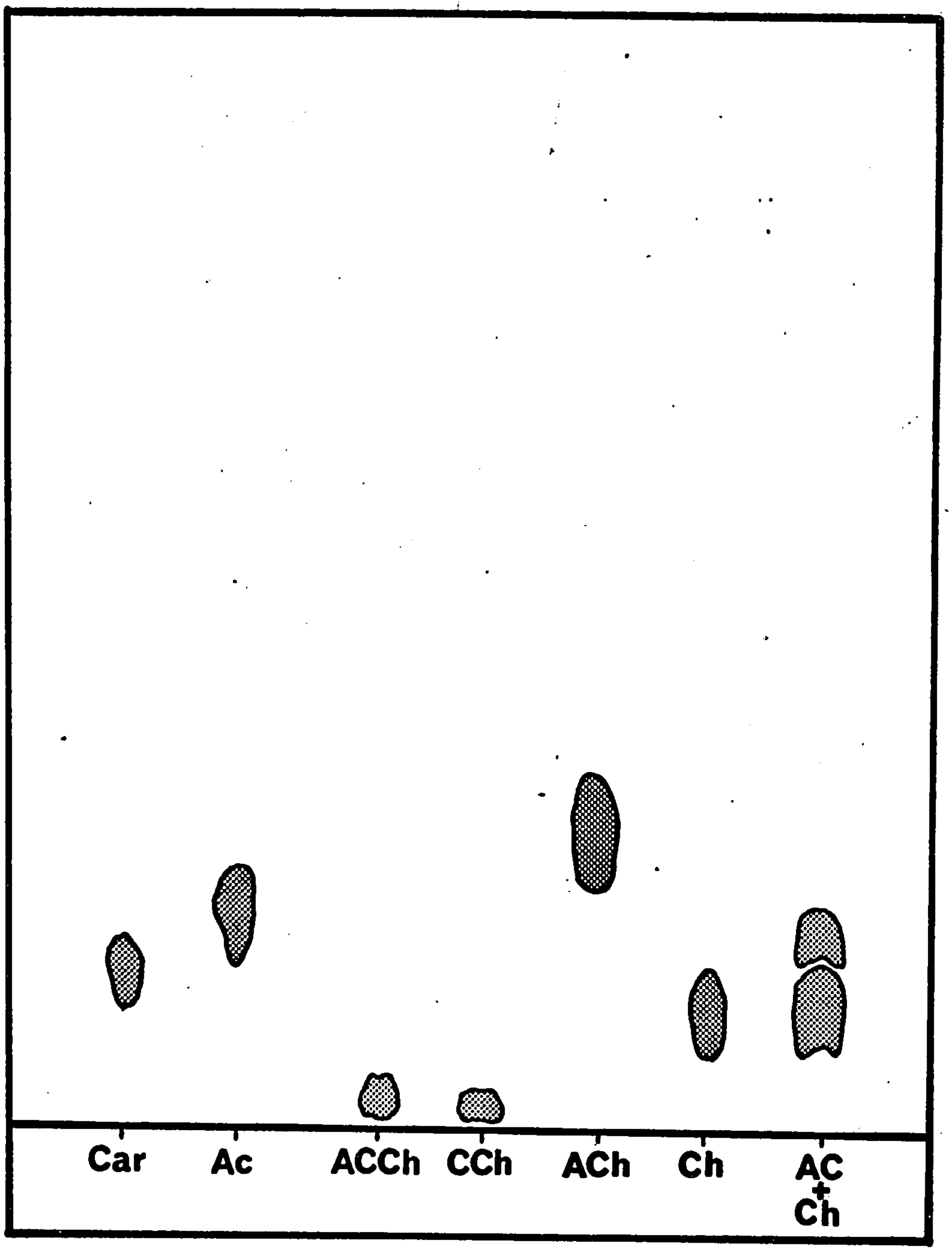

A
10
10 + E
15
15 + E
100
100 + E
100
100 + E
80
ACh (mμg)
l-ACCh (mμg)
3
2
1
3 + E
2 + E
l-CCh (μg)
B
8
6
6 + E
400
400 + E
400
400
400
1800
1800 + E
1000
1000
1200
1200
l-CCh (μg)
ACh (mμg)
l-ACCh (mμg)

Figure 34

Paper Chromatography of Carnitine and Choline Esters

Ascending chromatography was carried out according to Davies and Kalow (108) and Whittaker and Wijesundera (109). Whatman No.4 paper was employed in a solvent mixture of n-butanol: n-propanol: water (4:2:1). The development took place at room temperature for 18 hours. Approximately 100 µg of each pure standard compound in absolute methanol was applied to the paper with a micropipette. Iodine vapour was used to detect the spots.

Fig.34



$\underline{n}$-BuOH:$\underline{n}$PrOH:H_2O (4:2:1)

18 hours; room temperature; I_2 vapour

Table 11

Hydrolysis of dl-Acetylcarnitylcholine by Acetylcholinesterase

Time min	Active enzyme (50 μg)	Inact. enzyme	O.D. (Klett units)	ΔO.D.	% Hydrolysis
0	+	-	197;200	0	0
0	-	+	199;200		
1	+	-	194;195	5	2.5
1	-	+	200;201		
2	+	-	191;192	8	4.0
2	-	+	200;199		
4	+	-	181;182	18	9.0
4	-	+	199;199		
10	+	-	153;155	43	21.2
10	-	+	200;197		
15	+	-	148;148	50	25.2
15	-	+	198;197		

Incubation mixtures contained 1.2 μMoles dl-ACCh, 0.1 ml bovine erythrocyte AChE (0.5 mg/ml) made up in 0.1M sodium phosphate buffer, pH 7.0,to a total volume of 1.0 ml. The mixtures were incubated at 37°C for the specified times and the reaction terminated by the addition of the alkaline hydroxylamine colour reagents. The ester concentration in the mixtures was determined according to the modified Hestrin method (118). Controls for non-enzymatic hydrolysis of the substrate consisted of incubation in the presence of heat-inactivated enzyme, which had been incubated in a boiling water bath for 10 minutes.

different stages, four identical hydrolysis mixtures were employed. The course of the hydrolysis was stopped at 0,2,4, and 15 minutes by the addition of 0.1 ml eserine sulphate solution to give a final eserine concentration of $1 \times 10^{-5}M$. Aliquots of approximately 100 µl of each solution were pipetted onto the paper which was then developed in the standard chromatographic system given in Methods, Part B,3.

A summary of the resulting chromatograms is shown in Figure 35. The presence of protein caused tailing of the spots. The enzymatic breakdown of dl-ACCh in the presence of bovine erythrocyte AChE clearly gave rise to the end products choline and acetylcarnitine.

An attempt was made to reduce tailing by removal of the protein from solution. The enzymatic reaction was terminated by the addition of 9 ml ice-cold absolute methanol. The protein was precipitated, the solutions were centrifuged and the methanolic supernatant filtered into a 25 ml suction flask under vacuum. The methanol was evaporated, the residue dissolved in approximately 4 drops absolute methanol and applied to the chromatogram. Little or no improvement occurred in the tailing, essentially similar chromatograms being obtained.

6. Thin-Layer Chromatography (TLC)

The nature of the end products of the enzymatic hydrolysis of dl-ACCh was also studied using thin-layer chromatography. dl-ACCh, dl-AC,

Figure 35

Paper Chromatography of the End Products of Enzymatic Hydrolysis of dl-Acetylcarnitylcholine by Acetylcholinesterase

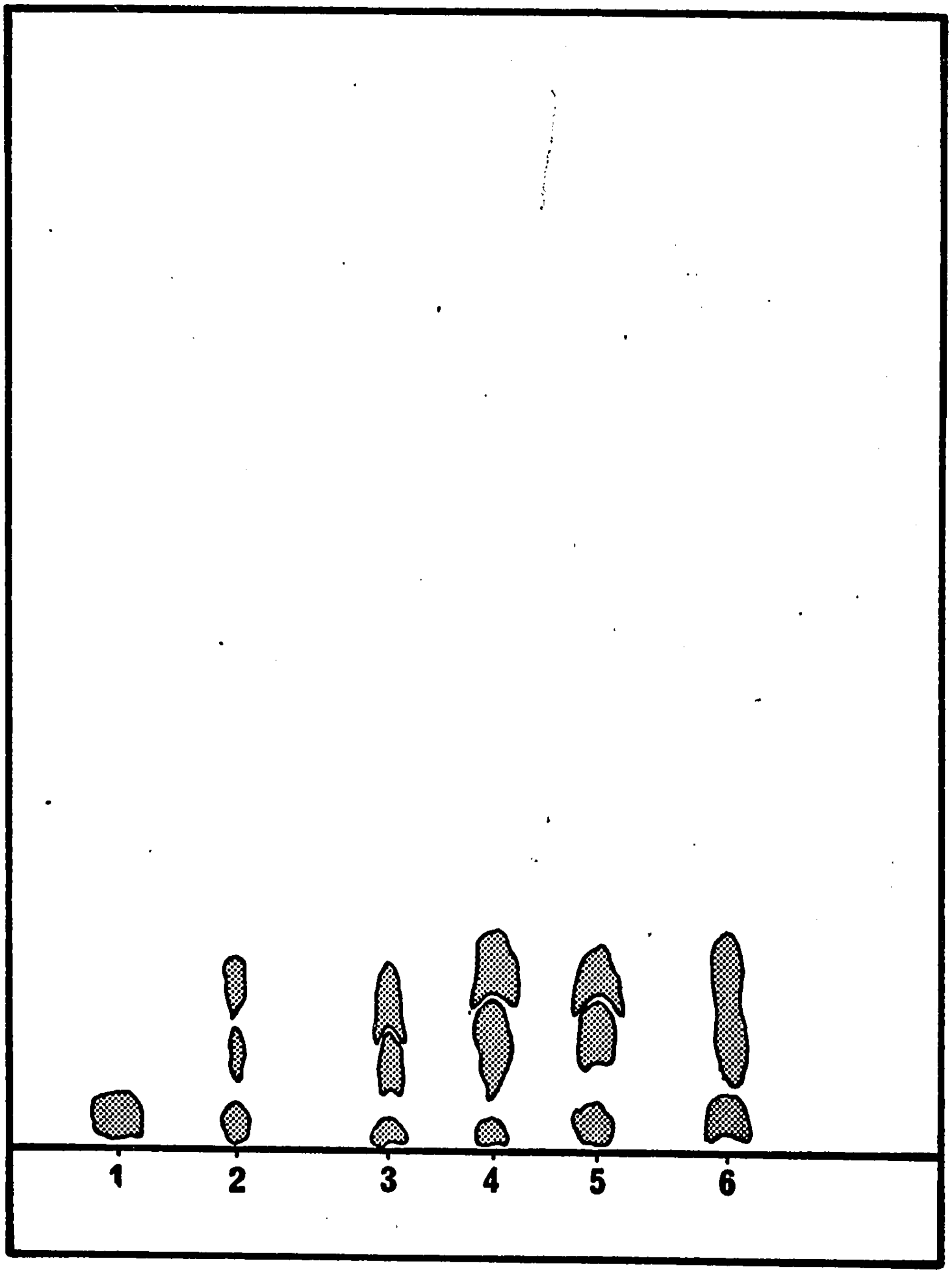
Ascending chromatography was carried out according to Davies and Kalow (108) and Whittaker and Wijesundera (109). Whatman No.4 paper was employed with a solvent mixture of n-butanol: n-propanol: water (4:2:1). The ascending chromatography was carried out at room temperature for 18 hours. Iodine vapour was employed for spot detection.

(1) - (4) Hydrolysates of dl-ACCh after 0, 2, 4, and 15 minutes of incubation in the presence of 0.1 mg bovine erythrocyte AChE (Sigma) at 37°C.

(5) Control mixture containing 100 µg each dl-ACCh, dl-CCh, dl-AC, and choline and 0.1 mg heat-inactivated AChE.

(6) Non-enzymatic hydrolysate of dl-ACCh incubated at pH 9.0 for 30 minutes.

Fig.35



n-BuOH; n-PrOH: H_2O (4:2:1)
18 hours; room temperature
I_2 vapour

and choline were each incubated with 1 mg bovine erythrocyte AChE (Sigma) in a total volume of 2.0 ml 0.1M sodium phosphate buffer, pH 7.0, for 60 minutes at 37°C to ensure total enzymatic hydrolysis. A control mixture for non-enzymatic hydrolysis contained dl-ACCh and enzyme which had been heat-inactivated by incubation for 10 minutes in a boiling water bath.

The reaction was terminated by the addition of 0.5 ml saturated trichloroacetic acid solution in an ice bath. After 10 minutes, the mixture was centrifuged at 10,000 x g for 15 minutes in the HR-1 International centrifuge at 4°C in order to remove the precipitated protein. The supernatants were then repeatedly extracted with 10 ml aliquots ice cold diethyl ether until the pH of the solutions was 4 to 5. The solutions were frozen in a dry ice-acetone mixture and lyophilized. They were taken up in absolute methanol, 20 µl were applied to a 100 µ thick silica gel G TLC plate and developed in a system of methanol/acetone/HCl (90/10/10, v/v/v). The plates were dried at room temperature and placed in iodine vapour to detect the spots.

The results of 6 experiments are presented in summary in Figure 36. The products of dl-ACCh hydrolysis appear to be ACCh, AC and choline. This indicated that only one isomer was hydrolyzed, since ACCh definitely was shown to be present in the mixture after 1 hour incubation, in the presence of 1 mg of a very active enzyme preparation. The control for non-enzymatic hydrolysis also showed traces of AC and choline although the

Figure 36

Thin-Layer Chromatography of the End Products of Hydrolysis of dl-Acetylcarnitylcholine by Acetylcholinesterase

A. The incubation mixture consisted of 5 mg dl-ACCh and 1 mg bovine erythrocyte AChE (Sigma) in 2.0 ml 0.1M sodium phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 60 minutes.

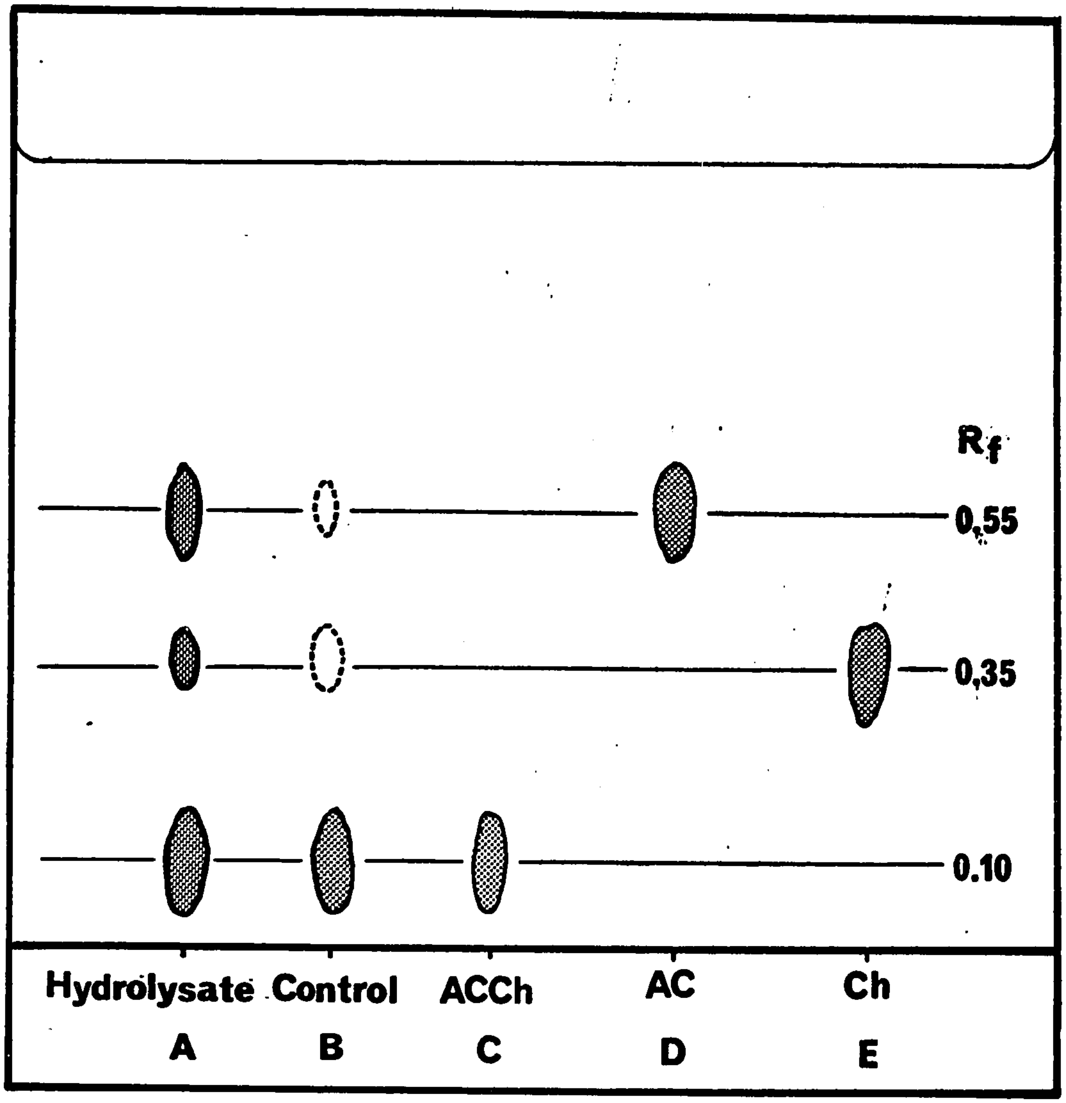
B. The incubation mixture consisted of 5 mg dl-ACCh and 1 mg heat-inactivated AChE in 2.0 ml 0.1M sodium phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 60 minutes.

C. Pure dl-ACCh

D. The incubation mixture consisted of 2 mg dl-AC and 1 mg AChE (Sigma) in 2.0 ml 0.1M sodium phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 60 minutes.

E. The incubation mixture consisted of 2 mg choline chloride and 1 mg AChE in 2.0 ml 0.1M sodium phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 60 minutes.

Fig.36



MeOH:Acetone:HCl (90/10/10, v/v)
Iodine vapour

presence of CCh could not be eliminated as it has the same R_f as ACCh in this solvent system.

7. High Voltage Electrophoresis

The nature of the end products of the hydrolysis of dl-ACCh by bovine erythrocyte AChE (Sigma) was investigated using high-voltage electrophoresis. dl-ACCh, dl-AC and choline were each incubated with 1 mg AChE in a total volume of 2.0 ml 0.1M sodium phosphate buffer, pH 7.0, at 37°C for 60 minutes to ensure total hydrolysis. A control mixture contained dl-ACCh and AChE which had been heat-inactivated by incubation for 10 minutes in a boiling water bath.

The reaction was terminated by addition of 0.5 ml saturated trichloroacetic acid solution in an ice bath. After 10 minutes, the mixture was centrifuged at 10,000 x g for 15 minutes in an HR-1 International centrifuge at 4°C in order to remove precipitated protein. The supernatants were then repeatedly extracted with 10 ml aliquots of ice-cold diethyl ether until a pH of 4-5 was reached. The solutions were frozen in a dry ice-acetone mixture and lyophilized. Then they were taken up in absolute methanol, 20 µl were applied to Whatman No.1 paper, and run at 1500 volts for 1.5 hours in an ammonium acetate buffer (0.5M, pH 4.6). The paper was dried at room temperature and placed in iodine vapour to visualize the spots.

The results of 5 experiments are summarized in Figure 37. The products of dl-ACCh enzymatic hydrolysis were ACCh, AC and choline (R_m values 1.25, 0.15 and 1.15, respectively), where

$$Rm = \frac{\text{distance moved by a compound}}{\text{distance moved by a standard}}, \text{ in this case ACh.}$$

The presence of significant ACCh in the hydrolysate supported the hypothesis that AChE attacks only one isomer of the racemic mixture. The presence of a trace amount of AC in the control mixture indicated that AC is also a product of non-enzymatic hydrolysis.

This high voltage electrophoresis separation appeared to be particularly useful for the separation of AC from other mono-quaternary compounds as it had a very low R_m value. This was thought to be due to partial neutralization of the positive charge on the quaternary nitrogen group by ring formation. The presence of AC and choline in addition to ACCh in the reaction mixture after treatment with acetylcholinesterase confirmed the previous results that ACCh is enzymatically hydrolyzed to AC and choline.

Figure 37

High Voltage Electrophoresis of the End Products of Hydrolysis of dl-Acetylcarnitylcholine by Acetylcholinesterase

I. Pure dl-ACCh chloride

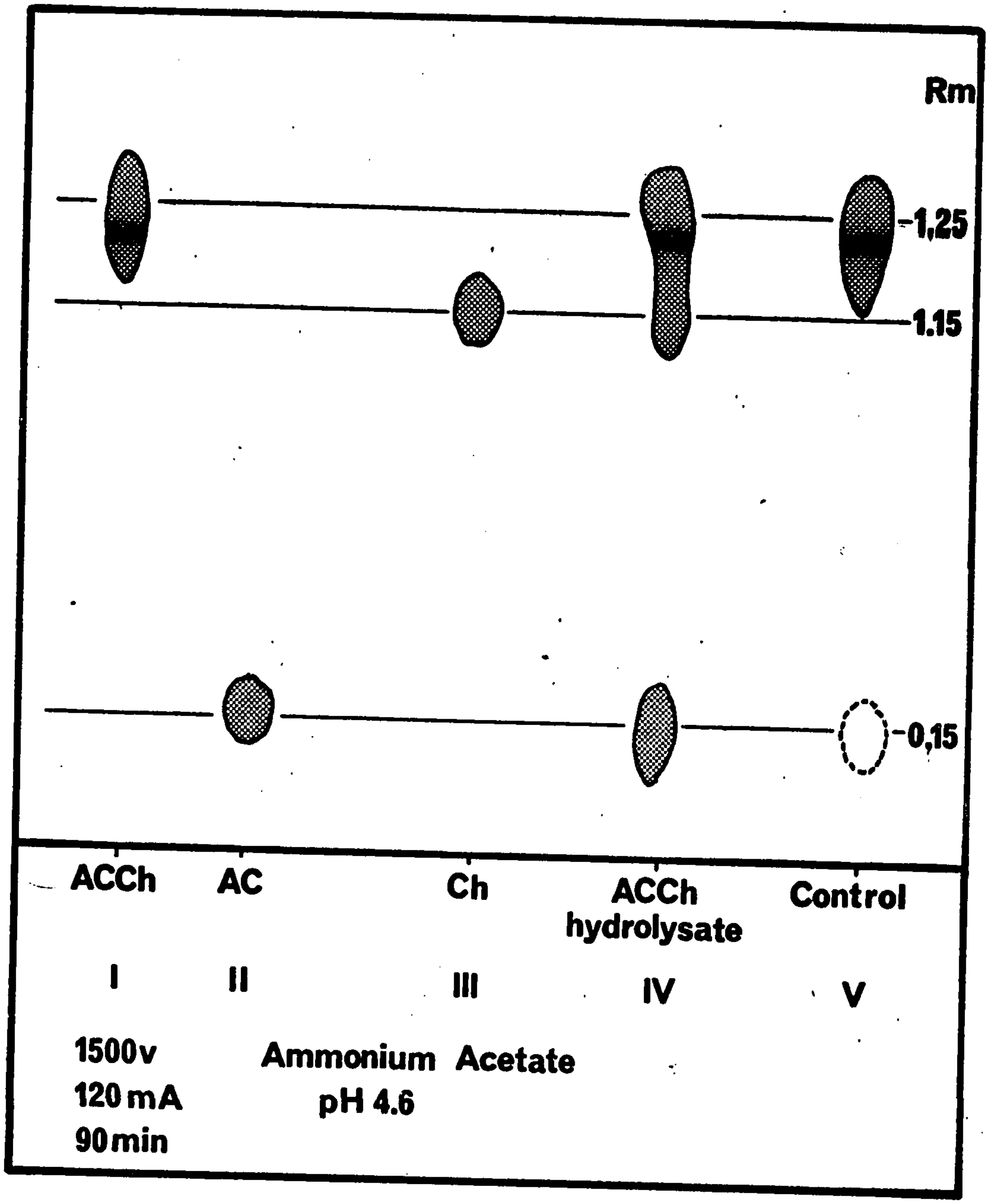
II. The incubation mixture consisted of 2 mg dl-AC and 1 mg bovine erythrocyte AChE (Sigma) in 2.0 ml 0.1M sodium phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 60 minutes.

III. The incubation mixture consisted of 2 mg choline chloride and 1 mg AChE in 2.0 ml 0.1M sodium phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 60 minutes.

IV. The mixture consisted of 4 mg dl-ACCh and 1 mg AChE in 2.0 ml 0.1M sodium phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 60 minutes.

V. The incubation mixture consisted of 4 mg dl-ACCh and 1 mg heat-inactivated AChE in 2.0 ml 0.1M sodium phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 60 minutes.

Fig.37


Rm
1,25
1.15
0,15
ACCh
AC
Ch
ACCh hydrolysate
Control
I
II
III
IV
V
1500v
120 mA
90 min
Ammonium Acetate
pH 4.6

DISCUSSION

Acetylcholine (ACh) was established as the cholinergic neurotransmitter of the body because its pharmacological actions mimicked those of nervous activity. Many findings, however, indicate that ACh may not be the exclusive cholinergic transmitter. Four specific facts have been advanced to support this hypothesis: (i) by analogy with the adrenergic transmitters, there is probably a group of related substances involved, (ii) ACh is widely distributed in nature, in both nervous and non-nervous tissues, (iii) many other choline esters and ACh-like substances have been isolated from animal tissues, and (iv) two classes of distinct cholinester hydrolases, differing as to specificity and stereochemical requirements, have been discovered.

Six criteria have been proposed by Paton (498) which must be fulfilled by a substance before it can be established as a synaptic transmitter. These are:

(i) The Criterion of the Presence of the Transmitter
(ii) The Criterion of the Collectability of the Transmitter
(iii) The Criterion of the Synthesizing Enzyme
(iv) The Criterion of Identical Effects
(v) The Criterion of Pharmacological Identity
(vi) The Criterion of the Inactivating Enzyme

Initially, the cholinergically active material of tissue extracts was considered to be ACh exclusively. In 1906, however, Kutscher and Lohmann (122) discovered carnitine, which was weakly active, in muscle extracts. In 1953, Banister _et al._ (48) demonstrated chromatographically the presence in alcohol extracts of ox spleen of PCh and a component 'F', both of which contributed a large fraction of the ACh-like activity and the chemical identification of PCh in spleen was reported in 1954 (55). BuCh was identified in ox brain by Holtz and Schumann (51) and other choline esters have been reported more recently (78). Cleugh _et al._ (499) and Shaw _et al._ (68) have sub-fractionated brain extracts into as many as 89 fractions and have found several to exhibit cholinergic activity.

Hosein _et al._ (53,54,131,132,139) have described the chromatographic and chemical identification, in rat brain extracts, of ACh, PCh, BuCh, and the CoA esters of GBB, crotonbetaine, carnitine and AC. In 1961, Kewitz identified GABA-choline in pig brain extracts (98). The discovery of all these esters has led to the proposal that other substances, besides ACh, may be involved in nerve functions (35,43,55,89,458,500). Recently acetyl-l-carnitylcholine (l-ACCh) has been chromatographically identified in rat brain extracts by three different methods. ACCh was of particular interest due to its physiological, pharmacological and chemical resemblance to ACh. ACCh is both a choline and a carnitine ester, contains within its structure two molecules of ACh and is a structural isomer of the muscle relaxant drug, succinyldicholine (SDCh).

Because of the trace quantities of transmitter substances released upon nerve stimulation into the perfusion medium, identification has generally been by means of bioassay techniques. It has been demonstrated, however, by Hosein and Koh (59,60) that pharmacological bioassay methods cannot identify a single substance in a mixture of active compounds. It is not unreasonable to assume, therefore, that the presence of l-ACCh and other compounds may have been overlooked in bioassay identification of tissue perfusates.

Emphasis has been placed on the fact that distribution of ACh and its metabolizing enzymes correspond. The natural occurrence, however, of several choline esters led to investigations into the specificity of choline acetylase. As pointed out in the Introduction, Nachmansohn et al. (63) demonstrated the synthesis by the choline acetylating system of rabbit brain of a biologically active acetate ester distinct from ACh. The enzyme, on further study, was found to be non-specific, synthesizing many choline esters including PCh, BuCh and pyruvylcholine. Hosein and Smoly (138) demonstrated the synthesis of l-ACCh using acetyl-l-carnityl CoA from narcotized rat brain extracts as substrate for a crude rat brain choline acetylase preparation.

Many choline esters have been found to exert cholinergic activity. l-ACCh was found to be one-half as active as ACh on the guinea pig ileum and frog rectus preparations (473). It also exhibits powerful cholinergic activity on the cat blood pressure, frog heart and rat phrenic nerve-diaphragm (471,472). On these preparations, l-ACCh and ACh were indistinguishable; both were inactivated by acetylcholinesterase (AChE) and alkali.

The criterion of pharmacological identity states that agents which interact with physiological synaptic agents will also interact with the proposed transmitter. The actions of l-ACCh were found to be potentiated by eserine (472) and abolished by atropine and d-Tubocurarine (471).

Finally, the criterion of the inactivating enzyme postulates that the transmitter, once it has produced its effects, must be rapidly destroyed and removed from the site of activity. The conclusion that this does, in fact, occur in vivo is based upon the presence in tissues of the cholinester hydrolases and on the evidence that, when these enzymes are inhibited by eserine or other anti-"cholinesterases", only then does "ACh" accumulate.

The existence of two distinct classes of cholinester hydrolases which markedly differ as to specificity led Pfeiffer (163,169) to suggest that choline esters of naturally occurring carboxylic esters might be present in animal tissues and that the cholinergic transmitter might be comprised of a group of compounds related to ACh. Since acetyl-ß-methylcholine appeared to be the most specific substrate for AChE, Pfeiffer proposed that the natural substrate for AChE should contain a ß-carbon substituent. AChE is known to exhibit stereochemical specificity. For example, the enzyme is very specific for D-acetyl-ß-methylcholine (382) and Ammon and Meyer (443) demonstrated that AChE of brain hydrolyzed the l-isomer of the choline ester of mandelic acid. The same relation held true for l-isomethadone (308), again with a ß-substituent. From such observations and structure-activity studies by Pfeiffer (163) and Beckett *et al.* (166), substances such as l-ACCh might be more natural substrates for AChE than ACh. l-ACCh, on the other hand, contains the BuCh backbone and is an isomer of SDCh. As such, it might be expected to be a substrate for ChE.

Many investigations have been carried out on the hydrolytic mechanism of AChE and the results have left some interesting questions unanswered. In its simplest form the reaction sequence of the hydrolysis of ACh by AChE is as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.S \xrightarrow{k_3} \begin{matrix} E' \\ + \\ P_1 \end{matrix} \xrightarrow{k_4} E.P_2 \xrightarrow{k_5} \begin{matrix} E \\ + \\ P_2 \end{matrix}$$

AChE has two spatially and functionally separated active sites. The anionic site attracts the quaternary nitrogen group; in the esteratic site, the electrophilic carbon of the carbonyl group of the substrate forms a covalent bond with the oxygen of a serine residue (501,502). As pointed out in the *Introduction,* there are imidazole rings of histidine residues in the vicinity for accepting the proton released in the hydrolytic process (503).

The forces involved in binding ACh or other substrates to the active surface of the enzyme to produce the Michaelis-Menten complex differ at the two centres. The major forces involved at the anionic site are coulombic (355,504), van der Waal's (424), and hydrophobic (467). The major force at the esteratic site is the formation of a weak covalent bond (504). An electronegative group "G": brings about a nucleophilic attack on the electrophilic carbonyl carbon atom of the substrate ester grouping.

If the substrate is molecularily complementary to the enzyme active surface (355), the individual forces act simultaneously and the substrate is bound to the active centre. If the molecule, on the other hand, is not complementary, as in the case of some of the optically active isomers of substrates, binding would be poor.

Initially the free enzyme and substrate are in dynamic equilibrium with the E.S. complex. It is probable, in fact, that normally the formation of the enzyme-substrate complex is the most important factor in the rapid removal of ACh, or another cholinergic transmitter, and that, providing the enzyme capacity is adequate, the actual hydrolysis of the complex is less important in terminating post-synaptic excitation (399). After formation of the Michaelis-Menten complex, acetyl-enzyme formation occurs with the splitting out of the first reaction product, choline (P_1), according to Wilson (451,504).

The acetyl group, or acetylcarnityl group, remains bound to the esteratic site (451,505), although the existence of the acyl enzyme is transient, i.e., 42 μsec according to Wilson and Harrison (434). Its existence, however, prevents another substrate molecule from being properly bound to the active site by steric interference (506).

AChE exhibits marked substrate inhibition when ACh is the substrate. Wilson and Cabib (427) explained this in terms of a two-step hydrolytic process. The acetyl enzyme might bind a second ACh molecule at the anionic site and this could then inhibit the hydrolysis of the acetyl enzyme. Although the acetyl-enzyme has never been isolated, acetylchymotrypsin has been (507).

Rapid restoration of the free enzyme requires that the acetyl group be split off. Estimates of a turnover number of 670,000 molecules ACh hydrolyzed/active centre/min for eel AChE (411), 300,000 molecule/min for ox red cell AChE (469), and 162,000 molecules/min for human erythrocyte AChE (514) indicate the speed with which this reaction takes place. A polar water molecule approaches the acetylated esteratic site (451) and the electronegative nucleophilic oxygen atom of the water attacks the electrophilic carbonyl carbon atom of the acetyl enzyme (451). An unstable complex forms by an SN_2 reaction which breaks down almost immediately to release acetic acid (508).

In addition to physical properties of the substrate molecule, molecular complementariness is an important factor in enzyme binding (355,365,367), as was strongly supported by the studies of Wilson and associates (349,515, 516) with a large number of phenolic and non-phenolic phenyltrimethylammonium salts.

Studies on the thermodynamics of the binding and hydrolysis of esters related to ACh have been used to suggest conformational changes in AChE after or during the binding of substrate. The great difference in the rates of enzymatic hydrolysis of ACh and its tertiary analogue, coupled with the discovery that this difference is entropic in origin, was the basis of this suggestion by Mautner (427,517).

Results obtained with inhibitors of AChE offered evidence that a quaternary nitrogen compound, having a hydroxyl group at a distance of approximately 5 Å from the nitrogen, forms a hydrogen bond with a basic atom, probably the oxygen of serine, in the esteratic site. The intramolecular distance seems, therefore, to be an important factor in reaction of molecules with the receptor and AChE (518).

Certain molecules, not possessing a quaternary ammonium group, react with the esteratic site alone, leaving the anionic site free. Such compounds as dimethylcarbamyl fluoride and methanesulphonyl fluoride react with the esteratic site to form inactive acyl-enzymes. Their rates of acylation are accelerated by as much as 30 times in the presence of small alkyl ammonium ions (519). According to Metzger and Wilson (520) the binding of the quaternary ammonium ion induces a change at the ester site which causes this site to react more rapidly with the acid fluoride. This kind of explanation is in agreement with the concept of "induced fit" of Koshland (521) who has emphasized the importance of conformational changes in the combination of substrate and enzyme (522).

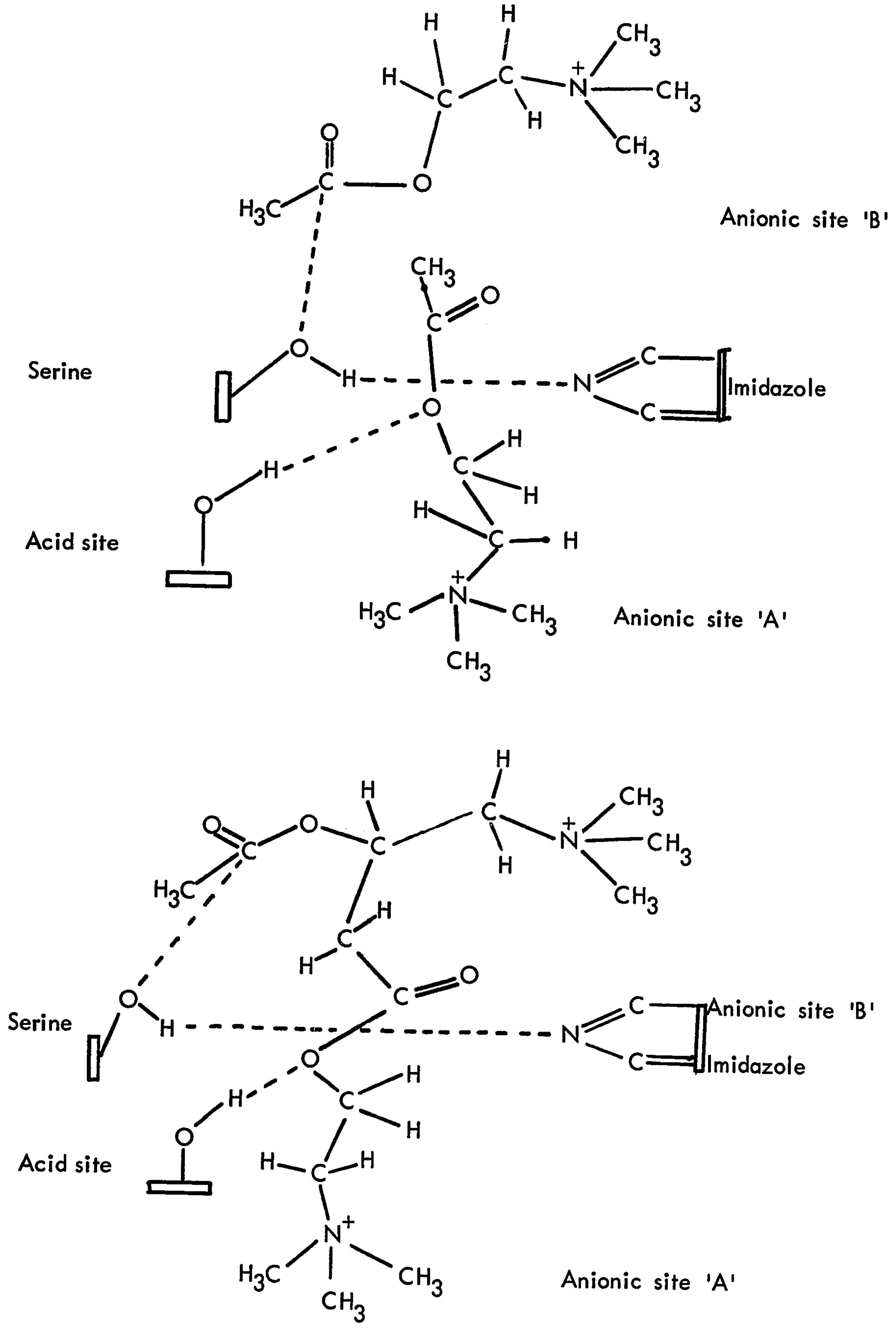
Brestkin and Rosengart (523) have suggested that ACh actually participates in the activation of the catalytic centre of AChE. Their scheme proposes that, as a result of the adsorption of ACh to the active centre due to interaction of the quaternary nitrogen and anionic site, hydrogen bonding takes place between the carbonyl oxygen of ACh and the histidine imino-nitrogen. This bond formation causes a rearrangement of the active centre which permits the secondary interaction of the activated azolic nitrogen and a neighbouring serine hydroxyl oxygen. This would result in a nucleophilic attack by the serine hydroxyl on the carboxyl group of ACh. Release of the choline molecule leaves the acylated enzyme. A similar interaction would then lead to the formation of new bonds between the acylated enzyme and a water molecule.

More recent investigations by Krupka (524,525,526) have indicated that the enzyme active site is considerably more complex than had been previously proposed. He found that two enzyme groups that ionize on the acid side of the pH optimum affect substrate hydrolysis by bovine erythrocyte AChE. One ($pK = 6.3$) plays no essential role in substrate binding or in reaction of the enzyme-substrate complex, but when protonated, prevents cationic substrates from becoming bound to the active centre. The other ionizing group ($pK = 5.5$) is an essential component of the catalytic mechanism (524). These two basic groups are catalytically active when unprotonated.

The one group (pK = 5.5) is thought to function in acetylation and is apparently located at least 9 Å from the anionic site, while the other (pK = 6.3) functions in deacetylation and is situated approximately 5 Å from the anionic site. Krupka reported (525) that the anionic site is neutralized when this group (pK = 6.3) is protonated. The anionic site is protonated itself at pH 4.0 - 4.5 and is, therefore, most probably a carboxyl side-chain of an aspartic or glutamic acid residue. A fourth group (pK = 9.2), which has been found to function in the protonated form, is more than 10 Å from the anionic site (525). Krupka has recently also presented evidence that an intermediate, whose formation and decomposition are catalyzed by different enzyme groups, must occur in acetylation (526). Many of these proposals have been necessary in order to explain, in some way, why ACh should interact with an enzyme active centre containing four separate active sites, including two anionic groupings. Many of the problems and questions could be explained if a bis-quaternary diester such as ACCh were the naturally occurring substrate of AChE.

The results of Beckett's experiments (342) with muscarines, muscarones, dioxolanes, and acetyl-methylcholines implicated a common muscarinic receptor which not only possessed an anionic site to associate with the quaternary group of all these compounds, but also a group in the receptor which would interact with alcoholic OH groups, ketones, ethers and the carbonyl of esters. Beckett (342), on consideration of the stereoselectivity of the acetyl-ß-methylcholine isomers, modified the proposed active site of AChE given by Krupka and Laidler (527). This proposed active site also possessed two anionic sites which interact with the quaternary nitrogen groups of 2 substrate molecules while the hydroxyl oxygen of the first substrate molecule, interacting with anionic site 'A', was hydrogen bonded to the acid site and the carboxyl carbon of the second substrate molecule to the activated serine OH oxygen. The two substrate molecules in this proposed active site could equally well be replaced by a single molecule of ACCh which could interact with the four sites simultaneously. This would relieve the necessity of proposing the involvement of two substrate molecules in the activation of a single enzyme active centre. Beckett's (342) proposed active site is presented in the diagram below. An interesting feature of this active site is the indication it offers that one might not get substrate inhibition with ACCh whereas you might with ACh as substrate. The possibility of substrate inhibition by ACCh remains to be explored. Certainly, all the evidence concerning the stereochemical specificity of AChE and the presence of the two anionic sites within the active centre was strongly indicative of the possibility that the simple molecule, ACh, might not be the natural substrate for AChE.

The hydrolysis of dl-ACCh, therefore, by these enzymes, was investigated. The method employed for the majority of the determinations of enzymatic hydrolysis was that of Hestrin (477), modified by Friedman



Proposed active site of acetylcholinesterase (342) and Fraenkel for carnitine derivatives (478, 118). The merit of the colorimetric method is its adaptability to widely different experimental conditions. The reaction kinetics of the esterases could be studied by this method with a uniform procedure over a wide range of pH's, substrate and enzyme concentrations, in almost any buffer system.

The standard manometric assay for cholinester hydrolases was not suited to the study of dl-ACCh hydrolysis due to excessive non-enzymatic hydrolysis in the buffer system employed and the comparatively large concentrations of substrate required. The availability of ACCh was strictly limited and the method, therefore, was not employed for these studies.

As seen in the Results, dl-ACCh was not hydrolyzed by purified horse serum cholinesterase (ChE) (Sigma) and only to a slight and variable extent by highly active human serum ChE (Schwarz). This was not unexpected in view of the lack of stereospecificity of this enzyme although it does hydrolyze the structural isomer, SDCh.

The function of ChE remains unsolved. The true substrate for this enzyme is unknown. It hydrolyzes SDCh, an isomer of ACCh, but exhibits little activity toward the latter. The shapes of these molecules differ considerably, SDCh being a linear bis-quaternary compound, whereas ACCh exists in a "Y" shape. Thus, it is not surprising that they are not hydrolyzed by the same enzyme.

dl-ACCh was, on the other hand, hydrolyzed by the AChE's of rat brain, human and bovine erythrocytes. For ACh, total hydrolysis was obtained with 100% decrease in the concentration of ester bonds in solution. For dl-ACCh, however, the maximum enzymatic hydrolysis obtained was never greater than approximately 25% under any conditions employed, even incubation at 37°C for 4 hours.

The discovery of the hydrolysis of dl-ACCh by the isolated crude cholinester hydrolases of rat brain, fresh human erythrocytes and purified bovine erythrocyte AChE indicated that the hydrolysis was not due to fortuitous species or tissue specificity. Investigation of a variety of animal tissues for ACCh hydrolytic activity remains to be carried out as additional substrate becomes available.

It is proposed that this value of 25% represents total hydrolysis of a single ester bond in one of the diester enantiomorphs, that is, one of the four ester bonds present in two molecules of acetyl-dl-carnitylcholine. This hypothesis is supported by the demonstrated stereospecificity of AChE and the fact that l-carnitine is the naturally occurring isomer. This possibility could only be studied if sufficient of the isomers were available for enzymatic studies. This is not the case, and consequently, only the racemic mixture has been investigated.

The apparent K_m' of 4×10^{-3}M is of limited significance in light of the racemic nature of the substrate and is at least several times larger than the true value for the substrate. Another factor to be considered is the possible inhibition of AChE by the unhydrolyzed isomer. Although this factor could not be investigated, it has been documented that, in the presence of D(-)-lactoylcholine, the hydrolysis of both L(+)-lactoylcholine and ACh by human erythrocyte AChE is inhibited (380). The same type of inhibition by L-acetyl-ß-methylcholine was studied by Hoskin (383) who emphasized the presence of a 50:50 mixture of substrate and inhibitor in the available racemic substrate mixture. Another factor to be considered is that the concentration of inhibitor increases in such a mixture in direct proportion to the degree of hydrolysis of the substrate.

All the choline and carnitine esters investigated proved to be competitive inhibitors of AChE, ranging from strong to very weak in inhibitory strength. In view of the existence of two anionic sites in the active centre of this enzyme, it might be expected that any quaternary compound would exert some inhibitory effect. It was noteworthy, however, that dl-ACCh was found to cause very strong inhibition, indicating an affinity for the active centre at least equal to that of ACh in this system. While it is interesting to speculate on the significance of the inhibition of AChE by these quaternary choline and carnitine esters, it should be recalled that the measurements were carried out in a standard incubation mixture containing Tris buffer which has itself been shown to inhibit the enzyme (401). Thus the K_i' values obtained actually represented total inhibition in the mixture and were primarily of significance relative to one another. In the case of dl-ACCh, the relative contribution to the inhibitory effect made by the individual isomers remains to be investigated as soon as the purified isomers become available. It was found that the two CCh derivatives were fairly weak inhibitors of the enzyme, despite their close resemblance to their acetylated derivative, indicating the specificity of interaction of the enzyme active centre with ACCh. The proven existence in tissues of ACh, PCh, ACCh, and possibly Ac-ß-MeCh, all of which have very strong affinity for the enzyme centre according to these findings, indicated possibility of inhibitory controls over the activity of this ubiquitous enzyme.

Much fundamental information about the structure of the active centres in cholinester hydrolases has been derived from investigations of the pH dependence of the activity of the enzymes. The AChE of bovine erythrocytes exhibited maximum activity in the range pH 7 to 8.5. Bergmann et al. (509) showed that eel AChE equilibrated with the OH^- ions of the incubation medium so rapidly that the length of the incubation period had no influence upon hydrolytic rates. They concluded that the shape of the pH activity curve in the alkaline range must represent rapid changes at the active surface involved in the hydrolytic process. Thus the greater the affinity of the substrate for the electrophilic group of the active centre, the more rapid the decrease in rate of enzymatic hydrolysis at higher pH's. Based on this reasoning, the shape of the pH-activity curve for AChE hydrolysis of dl-ACCh indicated strong affinity of the substrate for the enzyme centre.

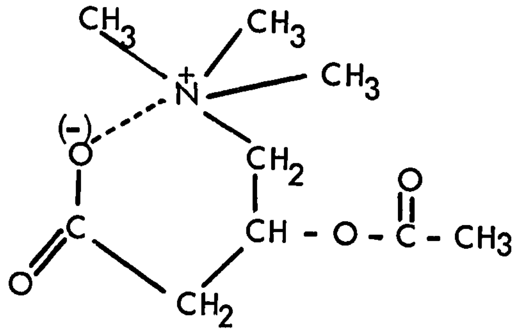
The energies of activation of enzymatic hydrolysis of ACh and dl-ACCh were similar, as expected for the reactions by a single enzyme (510). The low activation energy of approximately 4000 cal/g mole, however, was smaller than those reported for ordinary enzymes (511). As mentioned previously, Wilson and Cabib (427) have reported a strong entropic component in the hydrolysis of ACh which may account for this low activation energy. The same may well hold true for dl-ACCh.

The nature of the end products of enzymatic hydrolysis of dl-ACCh was investigated in several systems described in the section on Results. Direct identification of the products was very difficult due to the similarities of the chemically reactive groups possibly present in the reaction mixtures. In particular, all the compounds, with the exception of acetic acid, possessed quaternary nitrogen groups which interfered with the identification of the released choline both chemically and enzymatically. No chemical test was specific for acetylcarnitine in the presence of choline and ACCh and consequently the stoichiometric nature of the reaction could not conclusively be demonstrated with the racemic mixture.

The inhibition of choline dehydrogenase by carnitine esters of the nature under consideration has not previously been reported, although trimethylammonium compounds were known to inhibit this enzyme (485). This might represent a metabolic control system affecting choline metabolism. It did, however, result in the inability of this enzyme to identify choline in a mixture of choline and carnitine esters.

The loss of only 25% of the ester bonds from the reaction mixture during enzymatic activity indicated that only one ester bond of the diester was hydrolyzed. This meant that either an acetyl group or a choline molecule was removed during hydrolysis. Because AChE has been found to be relatively specific for acetate esters, as its name implies, it was somewhat unusual to find that acetate was not split from the molecule.

Investigation of the possible hydrolysis of acetylcarnitine (AC) by AChE and ChE showed absolutely no cleavage of the acetate bond. In view of the previous findings that very few acetate esters were not hydrolyzed (167), it was concluded that AC does not have the proper steric configuration to interact with the enzyme. This was supported by the discovery that dl-AC was an extremely weak inhibitor of AChE although, on the basis of its chemical structure alone, it would be expected to be a relatively strong competitive inhibitor. Fellman <u>et al.</u> (369) proposed a cyclic conformation for AC, since the positive charge on the quaternary nitrogen can interact with the negatively charged carboxylate group by an ion-dipole bonding shown below.



This proposed cyclic structure would bring about neutralization of the positive charge on the quaternary head, which is required for enzyme-substrate complex formation by the cholinester hydrolases. This theory of ring formation was borne out by the low mobility of AC in a strong electrical field found in the high voltage electrophoresis experiments.

Studies of the attempted hydrolysis of l-carnitylcholine showed no hydrolysis by either cholinester hydrolase. This was of interest in light of Foldes' finding (357) that the deoxycarnitylcholine derivative, GBB-choline, was not hydrolyzed by either enzyme. It did, however, present an example of marked enzyme specificity. dl-ACCh was well hydrolyzed, the products being AC and choline, whereas the removal of the acetyl group completely abolished enzymatic destruction of the carnitine-choline ester bond. This marked specificity may be significant physiologically. l-Carnitylcholine has been shown to be a powerful neuromuscular blocking agent (475). If it were to arise within an organism, the symptoms of neuromuscular block would appear with serious consequence to the animal involved.

Experiments presented in this thesis have demonstrated that the products of normal hydrolysis of dl-ACCh are AC and choline. Further indirect evidence has been available in our laboratory to support this conclusion. Studies on the frog heart preparation (471) showed that the ACCh-type block did not change with time to a CCh-type block. Eserine potentiated the actions of ACCh without having any effect upon the activity of CCh (471) and injection of ACCh into day-old chicks produced transient neuromuscular blockade which gradually disappeared with total recovery whereas l-CCh blocking action was prolonged.

In view of the proposal that l-ACCh may have a role as a cholinergic neurotransmitter, the finding that the products of ACCh hydrolysis are essentially inactive when assayed for nicotinic and muscarinic cholinergic activity is of particular significance.

Hunt and Taveau reported in 1911 (528) that the acetyl esters of formocholine and homocholine were very much less active than ACh, and several investigators have demonstrated the importance of structural and stereochemical features of cholinergic compounds (163,164). Recent experiments on the isolated electroplax preparation demonstrated the unique lack of depolarizing activity of choline which was found to be 33,000 times less active than ACh (211). Webb and Mautner (211) concluded that the specificity of ACh action at the synapse was dependent to a large extent upon the nature of its hydrolysis product, choline, which fails to depolarize the post-synaptic membrane. This same specificity would hold true for l-ACCh which gives rise, upon hydrolysis, to choline.

The other end product of the reaction is AC, another molecule which exhibits certain unique properties. The isolation of carnitine from mammalian muscle (113) led to studies of its significance in metabolism. In 1948, Fraenkel discovered a growth factor for the larva of the beetle, *Tenebrio molitor,* which he named vitamin B_T and which was subsequently identified as carnitine (529). Later studies of liver extracts showed that carnitine was reversibly acetylated to AC (118).

The role of acetyl-CoA in biological systems has been studied and is well known but the number of compounds which can donate acetyl groups to coenzyme A is limited due to the high energy of the ester bond of acetyl-CoA. AC can transfer its acetyl group to CoASH, unlike ACh, because of the high group potential of its ester bond. The properties, however, which give rise to this energy level have not been accounted for. Friedman and Fraenkel (118) proposed that the energy state is a function of the nature of the secondary ester bond and its neighbouring quaternary ammonium group although in what way they did not specify. On the basis of this reasoning, the energy level of the acetate ester bond in ACCh would also be expected to be high, a point worthy of further investigation. In this regard, the apparent lack of acetylation of the secondary hydroxyl group of l-carnitylcholine by carnitine acetyltransferase (475) was noted.

The apparent equilibrium constant was 0.6 at pH 7.0 and 35°C for the reaction

$$\text{acetylcarnitine} + \text{CoASH} \rightleftharpoons \text{acetyl-CoA} + \text{carnitine}$$

on the assumption that only the l(–)-isomer was enzymatically active (155). Fritz *et al.* found that acetyl-CoA, propionyl-CoA, and butyryl-CoA reacted *in vitro* at approximately the same rates with carnitine acetyltransferase.

Recently the discovery in animal tissues of propionyl-, crotonyl-, and butyrylcarnitine has been reported (513). In fact, 20 to 25% of the total liver carnitine in rabbit liver was propionylcarnitine. This recalled Banister *et al.*'s (48) discovery that 25% of the "ACh" of ox spleen was in reality PCh and once again indicated the necessity of rigorous re-examination of the identification of bioactive quaternary nitrogen compounds in tissue extracts.

Evidence for the nature of the hydrolytic reaction end products, AC and choline, was obtained in several systems including paper and thin-layer chromatography and high voltage electrophoresis. The results were identical in all systems studied and appear to be consistent with the overall metabolic pathway proposed by Hosein (54), and recently extended to include a possible biosynthesis of acetyl-l-carnitylcholine as follows (138):

Protein → glutamic acid → GABA → GABA-CoA → GBBCoA →
crotonylbetainylCoA → l-carnitylCoA → acetyl-l-carnitylCoA →
acetyl-l-carnitylcholine → acetyl-l-carnitine + choline
↓ ↓
fatty acid metabolism lipid metabolism

In view of this proposal, several features may be examined. This pathway recalls that of fatty acid oxidation and it is of particular interest in light of the recent report of Bohmer and Bremer (513) of the isolation of crotonylcarnitine, propionylcarnitine and butyrylcarnitine from animal tissues, corresponding to the parallel proposal that PCh and BuCh arise from propionyl-CoA and butyrylCoA (48). Perhaps a whole range of choline and carnitine esters of these CoA derivatives remain to be isolated from tissues, their relative concentrations varying according to the metabolic state of the tissue in question. This concept receives some support in view of the discovery also of GABA-choline in pig brain (98).

ACCh and its end products offer an advantage over ACh as a chemical transmitter substance. It has been demonstrated that ACCh possesses very strong physiological activity, which is rapidly destroyed by an enzyme present in nerve and other tissues. Since AC, a hydrolytic reaction product, is a high energy compound, this substance could be regarded as a store of energy which could furnish the energy required for physiological activity at the site of transmission. The high group potential of the O-acetyl ester bond of AC and possibly of ACCh itself is not found in the O-acetyl bond of ACh. Release of AC during hydrolysis, would produce a localized energy source which could then be instrumental in propagating neuronal activity. AC could also be a fine control mechanism for the total metabolism of the activated neurone.

Thus comparisons between ACh and ACCh indicate the similarities between these compounds and show that ACCh fulfils the requirements for chemical transmitter substances. In particular, it appears to fulfil several of the specifications for an excellent substrate for "acetyl"-cholinesterase.

SUMMARY

(1) The hydrolysis of acetyl-dl-carnitylcholine by the cholinester hydrolases was investigated. The cholinester hydrolases of fresh rat brain subcellular fractions, fresh human erythrocytes and bovine erythrocyte acetylcholinesterase (Sigma) hydrolyzed dl-ACCh at 37°C to the extent of 25 per cent. This level of hydrolysis was consistent even with prolonged incubation in the presence of active enzyme. Because the assay method employed measured disappearance of ester bonds from the reaction mixture, it was concluded that total hydrolysis consisted of specific cleavage of a single ester bond in one of the diester enantiomorphs. The apparent K_m' ($4 \times 10^{-3}M$) of dl-ACCh hydrolysis by bovine erythrocyte acetylcholinesterase, therefore, was several times greater than the true value.

(2) Hydrolysis of dl-ACCh by horse serum cholinesterase (Sigma) did not occur and the hydrolysis by highly purified human serum cholinesterase (Schwarz) was limited and variable, while no hydrolysis was found with fresh human plasma enzyme in 30 minutes, at 37°C.

(3) No hydrolysis of acetyl-dl-carnitine or l-carnitylcholine was found with rat brain subcellular fractions, fresh human blood cholinester hydrolases, bovine erythrocyte acetylcholinesterase (Sigma) or horse serum cholinesterase (Sigma).

(4) Alkaline hydrolysis was more pronounced with the bisquaternary than the monoquaternary esters studied.

(5) The pH optimum of acetyl-dl-carnitylcholine hydrolysis by bovine erythrocyte acetylcholinesterase was 7.0 to 8.5, as found for acetylcholine as substrate.

(6) The energy of activation for acetylcholinesterase hydrolysis of acetylcholine and acetyl-dl-carnitylcholine was 4,211 and 3,800 cal/mole, respectively.

(7) The inhibition of purified bovine erythrocyte acetylcholinesterase hydrolysis of acetylthiocholine iodide by choline and carnitine esters was investigated. All compounds studied inhibited the enzyme competitively. Acetyl-dl-carnitylcholine had a very strong affinity for the enzyme, the d- and l-carnitylcholine isomers were moderately strong inhibitors and choline and acetyl-dl-carnitine were very weak competitive inhibitors of acetylcholinesterase.

(8) Choline dehydrogenase did not oxidize carnitine derivatives, but was inhibited by several carnitine compounds.

(9) The "free" choline method of Appleton was shown to be non-specific.

(10) The products of enzymatic hydrolysis of acetyl-dl-carnitylcholine

were acetylcarnitylcholine, acetylcarnitine and choline. The hydrolysis appeared to be stereospecific, and very specific for this acetylated compound, as the closely related carnitylcholine was not hydrolyzed.

(11) The significance of acetyl-l-carnitylcholine as a possible neurotransmitter and the particular role of the hydrolytic end-product, acetylcarnitine, in neuronal metabolism were discussed.

CLAIMS TO ORIGINAL RESEARCH

1. Acetyl-dl-carnitylcholine chloride, a bisquaternary diester found in rat brain extracts, is hydrolyzed by rat brain cholinester hydrolases, fresh human erythrocyte acetylcholinesterase, and purified bovine erythrocyte acetylcholinesterase (Sigma). The hydrolysis occurs until 25 per cent of the ester bonds are cleaved, indicating a stereospecific reaction.

2. Acetyl-dl-carnitylcholine chloride is hydrolyzed only slightly by highly purified human serum cholinesterase (Schwarz), and is not hydrolyzed by fresh human plasma and purified horse serum (Sigma) cholinesterases.

3. All cholinester hydrolase preparations investigated (rat brain subcellular fractions, fresh human blood cholinester hydrolases, purified horse and human serum cholinesterases, and bovine erythrocyte acetylcholinesterase) failed to hydrolyze l-carnitylcholine.

4. Purified bovine erythrocyte acetylcholinesterase was inhibited competitively by acetyl-dl-carnitylcholine, d- and l-carnitylcholine, and acetyl-dl-carnitine. The inhibition by acetyl-dl-carnitylcholine indicated an affinity for the enzyme equal to that of acetylcholine.

5. The products of enzymatic hydrolysis of acetyl-dl-carnitylcholine include acetylcarnitylcholine, acetylcarnitine and choline. The hydrolysis appeared to be stereospecific and very specific for acetyl-dl-carnitylcholine, as the carnitylcholine ester bond was not hydrolyzed.

6. The significance of acetyl-l-carnitylcholine as a possible neurotransmitter is discussed and speculation concerning the role of acetylcarnitine in neuronal metabolism is presented.

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TABLE I

Standard Biuret Curve of Protein Concentration

Albumin	Optical Density (540 mμ)						
mg/ml	1	2	3	4	5	6	7
10	0.087	0.085	0.089	0.090	0.087	0.087	0.087
20	0.176	0.172	0.172	0.172	0.175	0.172	0.175
30	0.257	0.252	0.251	0.252	0.252	0.251	0.252
40	0.329	0.300	0.311	0.317	0.317	0.320	0.317
50	0.377	0.362	0.368	0.372	0.372	0.365	0.368

Standard bovine serum albumin was prepared in distilled water and aliquots were analyzed in a total volume of 1.5 ml after addition of 1.5 ml biuret reagent and incubation at 38°C for 15 min at 540 mμ in a Junior Coleman Colorimeter.

TABLE II

Alkaline Hydrolysis of Acetylcholine Iodide, Acetyl-dl-Carnitine Hydrochloride, and dl-Carnitylcholine Chloride

Substrate	Acetylcholine		Acetyl-dl-carnitine		
Time minutes	pH 7.0 K.U.	pH 9.0 K.U.	pH 7.0 K.U.	pH 9.0 K.U.	pH 12.0 K.U.
0	92	92	73	73	73
10	92	92	74	73	51
20	92	91	72	72	43
30	90	91	72	72	38
45	89	90	70	70	31
60	90	91	70	71	25

Substrate	dl-Carnitylcholine			
Time minutes	pH 7.0 K.U.	pH 7.5 K.U.	pH 8.5 K.U.	pH 10.0 K.U.
0	95	95	92	85
5	99	-	-	-
10	93	92	73	56
15	96	-	-	42
20	97	84	65	38
30	94	86	61	32
45	95	84	-	30
60	95	83	50	25

One μMole of the pure compounds was incubated at room temperature for the indicated time in a volume of 1.4 ml 0.1M sodium phosphate (pH 8.5-10.5), or 0.1M sodium carbonate-sodium bicarbonate solution (pH 11.0). The remaining ester was assayed by the modified Hestrin method (118).

TABLE III

Dependence of Acetylcholine Iodide Hydrolysis by Acetylcholinesterase on Enzyme Concentration

Enzyme conc.	Initial velocity (Δ µl/30 min)				
mg	1	2	3	4	Mean
0.053	41.6	42.1	41.3	40.0	41.2
0.106	79.5	86.3	75.8	81.2	80.7
0.160	108.9	119.1	103.7	115.0	111.7
0.213	144.4	150.8	140.6	149.2	146.5
0.320	215.6	224.6	217.2	220.4	219.4

The standard manometric method was employed (Methods, Part F, 6). Incubation took place in the presence of 0.01 g ACh Iodide and varying concentrations of AChE (Sigma) (4 mg/15 ml bicarbonate-Ringer's solution) at 37°C for 40 minutes. The values presented are the initial velocities in µl CO_2 released per 30 minutes determined by the least squares method.

TABLE IV

Dependence of Acetylcholine Iodide Hydrolysis by Acetylcholinesterase on Substrate Concentration

Substrate conc.	Initial velocity (▲ µl/30 min)					
mg	1	2	3	4	5	Mean
0.5		128	138	125	132	130.7
1.0	141		142.5	136	150.5	142.5
3.0				148	153	150.5
4.0	151			141	148.3	146.7
5.0	137.5		140			138.7
8.0	130		135	136.5	132.9	133.6
10.0	108	108.5	107.5		107.2	107.8
20.0	85	91.5	76.9	85	84	84.4
30.0	77	80	79			78.6
40.0	67	67		58		64.2

The standard manometric method was employed (Methods, Part F, 6). Incubation took place in the presence of 0.2 mg bovine erythrocyte AChE (Sigma) and varying concentrations of ACh Iodide at 37°C for 40 minutes. The values presented are the initial velocities in µl CO_2 released per 30 minutes determined by the method of least squares.

Legend to TABLES V and VI

Incubation mixtures contained 0.1 ml freshly prepared nuclear fraction of whole rat brain homogenate in 0.25M sucrose (900 x g; 15 min), varying concentrations of the substrate, ACh Iodide or dl-ACCh Chloride in a total volume of 1.0 ml containing 0.02M $MgCl_2$, 0.10M NaCl, and 0.01M potassium phosphate buffer, pH 7.2. Incubation was carried out at 37°C for 30 minutes and the total cholinester hydrolase activity determined according to the modified Hestrin method (118). The activity is presented as µMoles substrate hydrolyzed per mg protein per 30 min. Incubation in the presence of 3×10^{-5}M eserine sulphate abolished enzymatic activity. Controls for non-enzymatic hydrolysis consisted of incubation in the presence of enzyme which had been heat-inactivated at 80°C for 60 minutes. The results are the mean values of 5 experiments.

TABLE V

Hydrolysis of Acetylcholine Iodide by the Nuclear Subcellular Fraction of Fresh Rat Brain

ACh ml	Active Enzyme (0.1 ml)	Inact. Enzyme (0.1 ml)	10^{-5}M Eserine ml	mean O.D. K.U.	ACh conc. µMoles	ΔµMoles	Enzyme mg	ΔµMole/mg
0.025	-	+	-	41	0.446			
0.025	+	-	0.2	41	0.446			
0.025	+	-	-	19	0.206	0.240	0.9	0.266
0.050	-	+	-	72	0.782			
0.050	+	-	0.2	72	0.782			
0.050	+	-	-	29	0.315	0.467	0.9	0.518
0.075	-	+	-	94	1.021			
0.075	+	-	0.2	94	1.021			
0.075	+	-	-	55	0.597	0.424	0.75	0.565
0.100	-	+	-	124	1.349			
0.100	+	-	0.2	124	1.349			
0.100	+	-	-	65	0.706	0.643	0.9	0.715
0.150	-	+	-	178	1.935			
0.150	+	-	0.2	177	1.923			
0.150	+	-	-	132	1.434	0.501	0.75	0.668
0.200	-	+	-	228	2.480			
0.200	+	-	0.2	228	2.480			
0.200	+	-	-	175	1.902	0.578	0.9	0.642
0.250	-	+	-	282	3.061			
0.250	+	-	0.2	281	3.054			
0.250	+	-	-	252	2.740	0.321	0.75	0.428

TABLE VI

Hydrolysis of Acetyl-dl-carnitylcholine Chloride by the Nuclear Subcellular Fration of Fresh Rat Brain

ACCh	Active Enzyme	Inact. Enzyme	10^{-5}M Eserine	mean O.D.	ACCh conc.	ΔµMoles	Enzyme conc.	ΔµMole/mg
ml	0.1 ml		ml	K.U.	µMole		mg	
0.025	-	+	-	68	0.393			
0.025	+	-	0.2	68	0.393			
0.025	+	-	-	60	0.346	0.047	0.9	0.052
0.050	-	+	-	125	0.722			
0.050	+	-	0.2	125	0.722			
0.050	+	-	-	105	0.607	0.115	0.9	0.128
0.075	-	+	-	171	0.989			
0.075	+	-	0.2	171	0.989			
0.075	+	-	-	155	0.896	0.093	0.75	0.124
0.100	-	+	-	224	1.295			
0.100	+	-	0.2	222	1.283			
0.100	+	-	-	194	1.121	0.174	0.9	0.193
0.150	-	+	-	330	1.908			
0.150	+	-	0.2	330	1.908			
0.150	+	-	-	305	1.761	0.147	0.75	0.196
0.200	-	+	-	435	2.515			
0.200	+	-	0.2	435	2.515			
0.200	+	-	-	400	2.310	0.205	0.9	0.228
0.250	-	+	-	530	3.061			
0.250	+	-	0.2	530	3.061			
0.250	+	-	-	500	2.890	0.171	0.75	0.228

Legend to TABLES VII and VIII

Incubation mixtures contained 0.1 ml freshly prepared mitochondrial fraction of whole rat brain homogenate in 0.25M sucrose (10,000 x g; 20 min), varying concentrations of the substrate, ACh Iodide or dl-ACCh Chloride, in a total volume of 1.0 ml containing 0.02M $MgCl_2$, 0.10M NaCl, and 0.01M potassium phosphate buffer, pH 7.2. Incubation was carried out at 37°C for 30 minutes and the total cholinester hydrolase activity determined according to the modified Hestrin method (118). The activity is presented as μMoles substrate hydrolyzed per mg protein per 30 min. Incubation in the presence of 3×10^{-5}M eserine sulphate abolished enzymatic activity. Controls for non-enzymatic hydrolysis consisted of incubation in the presence of enzyme which had been heat-inactivated at 80°C for 60 minutes. The results are the mean values of 5 experiments.

TABLE VII

Hydrolysis of Acetylcholine Iodide by the Mitochondrial Subcellular Fraction of Fresh Rat Brain

ACh	Active Enzyme	Inact. Enzyme	10^{-5}M Eserine	mean O.D.	ACh conc.	ΔμMoles	Enzyme conc.	ΔμMole/mg
ml	0.1 ml		ml	K.U.	μMole		mg	
0.025	-	+	-	42	0.456			
0.025	+	-	0.2	42	0.456			
0.025	+	-	-	22	0.239	0.217	1.08	0.210
0.050	-	+	-	67	0.729			
0.050	+	-	0.2	67	0.729			
0.050	+	-	-	11	0.119	0.610	1.08	0.565
0.100	-	+	-	113	1.229			
0.100	+	-	0.2	113	1.229			
0.100	+	-	-	10	0.109	1.120	1.08	1.037
0.150	-	+	-	166	1.806			
0.150	+	-	0.2	165	1.793			
0.150	+	-	-	16	0.174	1.632	1.08	1.512
0.200	-	+	-	212	2.302			
0.200	+	-	0.2	211	2.293			
0.200	+	-	-	52	0.565	1.737	1.08	1.607
0.250	-	+	-	263	2.859			
0.250	+	-	0.2	262	2.847			
0.250	+	-	-	84	0.914	1.945	1.08	1.800
0.300	-	+	-	315	3.422			
0.300	+	-	0.2	315	3.422			
0.300	+	-	-	152	1.652	1.770	1.08	1.640

TABLE VIII

Hydrolysis of Acetyl-dl-carnitylcholine Chloride by the Mitochondrial Subcellular Fraction of Fresh Rat Brain

ACCh	Active Enzyme	Inact. Enzyme	10^{-5}M Eserine	mean O.D.	ACCh conc.	$\Delta\mu$Moles	Enzyme conc.	$\Delta\mu$Mole/mg
ml	0.1 ml		ml	K.U.	μMole		mg	
0.025	-	+	-	68	0.393			
0.025	+	-	0.2	68	0.393			
0.025	+	-	-	54	0.312	0.081	1.08	0.075
0.050	-	+	-	115	0.665			
0.050	+	-	0.2	115	0.665			
0.050	+	-	-	84	0.485	0.180	1.08	0.167
0.100	-	+	-	216	1.249			
0.100	+	-	0.2	216	1.249			
0.100	+	-	-	154	0.890	0.359	1.08	0.332
0.150	-	+	-	315	1.821			
0.150	+	-	0.2	315	1.821			
0.150	+	-	-	246	1.421	0.400	1.08	0.370
0.200	-	+	-	420	2.425			
0.200	+	-	0.2	420	2.425			
0.200	+	-	-	330	1.908	0.517	1.08	0.479
0.250	-	+	-	505	2.919			
0.250	+	-	0.2	505	2.919			
0.250	+	-	-	415	2.399	0.520	1.08	0.482
0.300	-	+	-	610	3.525			
0.300	+	-	0.2	610	3.525			
0.300	+	-	-	510	2.945	0.580	1.08	0.537

Legend to TABLES IX and X

Incubation mixtures contained 0.1 ml freshly prepared combined microsomal and supernatant fractions of whole rat brain homogenate in 0.25M sucrose, varying concentrations of the substrates, ACh Iodide or dl-ACCh Chloride, in a total valume of 1.0 ml containing 0.02M $McCl_2$, 0.10M NaCl, and 0.01M potassium phosphate buffer, pH 7.2. Incubation was carried out at 37°C for 30 min and the total cholinester hydrolase activity determined according to the modified Hestrin method (118). The activity is presented as µMoles substrate hydrolyzed per mg protein per 30 min. Incubation in the presence of $3 \times 10^{-5}M$ eserine sulphate abolished enzymatic activity. Controls for non-enzymatic hydrolysis consisted of incubation in the presence of enzyme which had been heated at 80°C for 60 minutes. The results are the mean values of 4 experiments.

TABLE IX

Hydrolysis of Acetylcholine Iodide by the Combined Microsomal and Supernatant Subcellular Fractions of Fresh Rat Brain

ACh	Active Enzyme	Inact. Enzyme	10^{-5} M Eserine	mean O.D.	ACh conc.	ΔμMoles	Enzyme conc.	ΔμMole/mg
ml	0.1 ml		ml	K.U.	μMole		mg	
0.050	-	+	-	59	0.641			
0.050	+	-	0.2	59	0.641			
0.050	+	-	-	15	0.163	0.478	1.13	0.423
0.100	-	+	-	111	1.207			
0.100	+	-	0.2	111	1.207			
0.100	+	-	-	16	0.174	1.033	1.14	0.906
0.200	-	+	-	210	2.281			
0.200	+	-	0.2	209	2.271			
0.200	+	-	-	49	0.533	1.748	1.13	1.547
0.300	-	+	-	315	3.422			
0.300	+	-	0.2	315	3.422			
0.300	+	-	-	143	1.555	1.867	1.14	1.638
0.400	-	+	-	405	4.400			
0.400	+	-	0.2	404	4.391			
0.400	+	-	-	241	2.620	1.780	1.10	1.620
0.500	-	+	-	500	5.440			
0.500	+	-	0.2	500	5.440			
0.500	+	-	-	330	3.585	1.855	1.10	1.685
0.300	-	+	-	560	6.090			
0.300	+	-	0.2	560	6.090			
0.300	+	-	-	390	4.240	1.850	1.11	1.666

TABLE X

Hydrolysis of Acetyl-dl-carnitylcholine Chloride by the Combined Microsomal and Supernatant Fractions of Fresh Rat Brain

ACCh ml	Active Enzyme 0.1 ml	Inact. Enzyme 0.1 ml	10^{-5}M Eserine ml	mean O.D. K.U.	ACCh conc. µMole	ΔµMoles	Enzyme conc. mg	ΔµMole/mg
0.05	-	+	-	64	0.370			
0.05	+	-	0.2	63	0.366			
0.05	+	-	-	57	0.329	0.041	1.13	0.036
0.10	-	+	-	115	0.665			
0.10	+	-	0.2	115	0.665			
0.10	+	-	-	86	0.495	0.168	1.114	0.147
0.20	-	+	-	206	1.190			
0.20	+	-	0.2	206	1.190			
0.20	+	-	-	165	0.954	0.236	1.13	0.209
0.30	-	+	-	323	1.865			
0.30	+	-	0.2	325	1.889			
0.30	+	-	-	250	1.445	0.420	1.14	0.368
0.40	-	+	-	420	2.425			
0.40	+	-	0.2	420	2.425			
0.40	+	-	-	325	1.878	0.547	1.10	0.497
0.50	-	+	-	510	2.945			
0.50	+	-	0.2	510	2.945			
0.50	+	-	-	410	2.367	0.578	1.10	0.525
0.30	-	+	-	620	3.582			
0.30	+	-	0.2	620	3.582			
0.30	+	-	-	500	2.890	0.692	1.11	0.623

TABLE XI

Hydrolysis of Acetylcholine Iodide by Fresh Human Erythrocyte Acetylcholinesterase

0.004M ACh	Act.	Inact.	10^{-5}M Eserine	mean O.D.	ΔO.D./30 min	Ester Hydrolyzed	
ml	Enzyme 10 µl			K.U.	ΔK.U.	µMoles	%
0.2	-	+	-	80			
0.2	+	-	+	80			
0.2	+	-	-	3	77	0.84	96.2
0.4	-	+	-	160			
0.4	+	-	+	160			
0.4	+	-	-	21	139	1.51	86.8
0.6	-	+	-	243			
0.6	+	-	+	242	1	0.01	0.4
0.6	+	-	-	81	162	1.76	66.6
0.8	-	+	-	323			
0.8	+	-	+	323			
0.8	+	-	-	131	192	2.08	59.4
1.0	-	+	-	400			
1.0	+	-	+	398	2	0.02	0.5
1.0	+	-	-	254	146	1.59	36.5

Incubation mixtures contained the indicated concentrations of substrate made up in a 0.1M sodium phosphate buffer, pH 7.0, 10 µl fresh enzyme prepared as described in Methods, Part F, 5, in a total volume of 1.0 ml made up in the phosphate buffer. Incubation was carried out at 37°C for 30 minutes and the amount of ester hydrolyzed was determined by the modified Hestrin method (118). The enzymatic activity was presented in change of optical density in Klett units/30 minutes. Controls for non-enzymatic hydrolysis consisted of incubation in the presence of enzyme which had been heat-inactivated by incubation in a boiling water bath for 10 minutes. The results are the mean values of 5 experiments.

TABLE XII

Hydrolysis of Acetylcholine Iodide by Fresh Human Plasma Cholinesterase

0.004M ACh	Act.	Inact.	10^{-5}M Eserine	mean O.D.	ΔO.D./30 min	Ester Hydrolyzed/30 min	
	Enzyme						
ml	10 µl			K.U.	ΔK.U.	µMoles	%
0.2	-	+	-	112			
0.2	+	-	+	112			
0.2	+	-	-	63	49	0.53	43.7
0.4	-	+	-	193			
0.4	+	-	+	192	1	0.01	0.5
0.4	+	-	-	124	69	0.75	35.8
0.6	-	+	-	276			
0.6	+	-	+	275	1	0.01	0.3
0.6	+	-	-	189	87	0.94	31.9
0.8	-	+	-	343			
0.8	+	-	+	343			
0.8	+	-	-	253	90	0.98	26.2
1.0	-	+	-	428			
1.0	+	-	+	427	1	0.01	0.2
1.0	+	-	-	335	93	1.01	21.2

Incubation mixtures contained the indicated concentrations of substrate made up in a 0.1M sodium phosphate buffer, pH 7.0, and 10 µl fresh enzyme prepared as described in Methods, Part F, 5, in a total volume of 1.0 ml made up in the phosphate buffer. Incubation was carried out at 37°C for 30 minutes and the amount of ester hydrolyzed was determined by the modified Hestrin method (118). The enzymatic activity was presented in change in optical density in Klett units/30 minutes. Controls for non-enzymatic hydrolysis consisted of incubation in the presence of enzyme which had been heat-inactivated by placing it in a boiling water bath for 10 minutes. The results are the mean values of 5 experiments.

TABLE XIII

Hydrolysis of Acetyl-dl-carnitylcholine Chloride by Fresh Human Erythrocyte Acetylcholinesterase

0.01M ACCh ml	Act. Enzyme 50 µl	Inact. Enzyme 50 µl	10^{-5}M Eserine	mean O.D. K.U.	ΔO.D./30 min ΔK.U.	Ester Hydrolyzed/30 min	
						µMoles	%
0.01	-	+	-	194			
0.01	+	-	+	193	1	0.01	0.5
0.01	+	-	-	146	48	0.28	24.7
0.02	-	+	-	388			
0.02	+	-	+	387	1	0.01	0.2
0.02	+	-	-	292	96	0.55	24.7
0.03	-	+	-	585			
0.03	+	-	+	584	1	0.01	0.1
0.03	+	-	-	436	149	0.86	25.4
0.04	-	+	-	355x2			
0.04	+	-	+	355x2			
0.04	+	-	-	266x2	178	1.03	25.0
0.05	-	+	-	445x2			
0.05	+	-	+	445x2			
0.05	+	-	-	349x2	202	1.17	22.7

Incubation mixtures contained the indicated concentrations of substrate made up in a 0.1M sodium phosphate buffer, pH 7.0, and 50 µl fresh enzyme prepared as described in Methods, Part F, 6, in a total volume of 1.0 ml made up in the phosphate buffer. Incubation was carried out at 37°C for 30 minutes and the amount of ester hydrolyzed was determined by the modified Hestrin method (118). The enzymatic activity was presented in change in optical density in Klett units/30 min. Controls for non-enzymatic hydrolysis consisted of incubation in the presence of enzyme which had been heat-inactivated by incubation in a boiling water bath for 10 min. The results are the mean values of 5 experiments.

TABLE XIV

Hydrolysis of Acetyl-dl-carnitylcholine Chloride by Fresh Human Plasma Cholinesterase

0.01M ACCh	Act. Enzyme	Inact. Enzyme	10^{-5}M Eserine	mean O.D.	Δ O.D./30 min	Ester Hydrolyzed/30 min	
ml	50 µl			K.U.	ΔK.U.	µMoles	%
0.01	-	+	-	194			
0.01	+	-	+	193	1	0.01	0.5
0.01	+	-	-	190	4	0.02	2.1
0.02	-	+	-	389			
0.02	+	-	+	389			
0.02	+	-	-	387	2	0.01	0.5
0.03	-	+	-	585			
0.03	+	-	+	583	2	0.01	0.3
0.03	+	-	-	582	3	0.02	0.5
0.04	-	+	-	354x2			
0.04	+	-	+	355x2			
0.04	+	-	-	354x2	-	-	-
0.05	-	+	-	443x2			
0.05	+	-	+	441x2	4	0.02	0.4
0.05	+	-	-	441x2	4	0.02	0.4

Incubation mixtures contained the indicated concentrations of substrate made up in a 0.1M sodium phosphate buffer, pH 7.0, and 50 µl fresh enzyme prepared as described in Methods, Part F, 6, in a total volume of 1.0 ml made up in phosphate buffer. Incubation was carried out at 37°C for 30 minutes and the amount of ester hydrolyzed was determined by the modified Hestrin method (118). The enzymatic activity was presented in change in optical density in Klett units/30 min. Controls for non-enzymatic hydrolysis consisted of incubation in the presence of enzyme which had been heat-inactivated by incubation in a boiling water bath for 10 min. The results are in the mean values of 5 experiments.

TABLE XV

Hydrolysis of Choline and Carnitine Esters by Purified Human Serum Cholinesterase (Schwartz)

Substrate Time	Butyrylcholine (3.0 µMoles)			Acetylcholine (3.0 µMoles)		
min	O.D.	ΔO.D.	% Hydrolysis	O.D.	ΔO.D.	% Hydrolysis
0	276	-	-	276	-	-
5	143	133	48.1	221	55	19.9
10	44	232	84.0	166	110	39.9
15	35	241	87.3	124	152	55.1
20	10	266	96.3	91	185	67.0
30	5	271	98.2	58	218	79.0
60	0	276	100.0	5	271	98.2
	Acetyl-dl-carnitylcholine (2.5 µMoles)			Succinyldicholine (2.5 µMoles)		
0	425	-	-	293	-	-
5	415	10	2.4	290	3	1.0
10	416	9	2.1	290	3	1.0
20	402	23	5.4	285	8	2.7
30	401	24	5.6	284	9	3.1
60	379	46	10.8	268	25	8.5

Incubation mixtures contained the specified concentrations of substrates and 60 µg human serum cholinesterase (Schwartz) in a total volume of 1.0 ml 0.1M sodium phosphate buffer, pH 7.0. Controls for non-enzymatic hydrolysis in the presence of heat-inactivated enzyme were subtracted from the enzymatic values obtained. Incubation was at 37°C for the specified time and the degree of enzymatic hydrolysis was measured by the modified Hestrin method. The values presented are the means of 10 determinations at each incubation time.

TABLE XVI

Hydrolysis of Acetylcholine Iodide and l-Carnitylcholine Chloride by Bovine Erythrocyte Acetylcholinesterase (Sigma)

Time min	ACh conc. μMoles	l-CCh conc. μMoles	Act. Enzyme (0.1 mg)	Inact. Enzyme (0.1 mg)	O.D. K.U.	ΔO.D. K.U.	% Hydrolysis
0	3.0	0	-	+	276		
2	3.0	0	+	-	175	101	36.6
5	3.0	0	+	-	85	191	69.0
10	3.0	0	+	-	42	234	84.8
30	3.0	0	+	-	2	274	99.3
0	0	3.0	-	+	254		
2	0	3.0	+	-	254	0	0.0
5	0	3.0	+	-	250	4	1.6
10	0	3.0	+	-	252	2	0.8
30	0	3.0	+	-	253	1	0.4

Incubation mixtures contained 3 μMoles ACh or 3 μMoles l-CCh and 0.1 mg bovine erythrocyte AChE (Sigma) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Enzymatic activity was determined using the modified Hestrin method (118). Controls for non-enzymatic hydrolysis at each incubation time consisted of incubation with heat-inactivated enzyme which had been incubated in a boiling water bath for 10 min. The values presented are the means of 8 determinations at each incubation time interval.

TABLE XVII

Hydrolysis of Acetylcholine Iodide and l-Carnitylcholine Chloride by Horse Serum Cholinesterase (Sigma)

Time min	ACh conc. µMoles	1-CCh conc. µMoles	Act. Enzyme (0.1 mg)	Inact.	O.D. K.U.	ΔO.D. K.U.	% Hydrolysis
0	3.0	0	-	+	275		
2	3.0	0	+	-	231	44	16.0
5	3.0	0	+	-	151	124	45.2
10	3.0	0	+	-	86	189	69.0
30	3.0	0	+	-	5	270	98.3
0	0	3.0	-	+	255		
2	0	3.0	+	-	254	1	0.4
5	0	3.0	+	-	252	3	1.2
10	0	3.0	+	-	253	2	0.8
30	0	3.0	+	-	251	4	1.6

Incubation mixtures contained 3 µMoles ACh or 3 µMoles 1-CCh and 0.1 mg horse serum cholinesterase (Sigma) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Enzymatic activity was determined using the modified Hestrin method (118). Controls for non-enzymatic hydrolysis at each incubation time consisted of incubation with heat-inactivated enzyme which had been incubated in a boiling water bath for 10 min. The values are the means of 6 determinations at each incubation time interval.

TABLE XVIII

Hydrolysis of Acetyl-dl-carnitylcholine (Horse Serum ChE)

Substrate conc. / Time minutes	1.0	2.0 (μMoles/ml)	3.0
	μMoles hydrolyzed ± SD		
0	$0.00_6 \pm 0.00_6$	0.019 ± 0.019	$0.00_0 \pm 0.00_0$
5	$0.00_0 \pm 0.00_0$	$0.01_9 \pm 0.01_9$	$0.03_0 \pm 0.00_0$
10	$0.02_5 \pm 0.01_9$	$0.00_0 \pm 0.00_0$	$0.00_0 \pm 0.00_0$
15	$0.00_0 \pm 0.01_9$	$0.01_9 \pm 0.01_9$	$0.06_1 \pm 0.03_0$
20	$0.01_9 \pm 0.01_2$	$0.01_9 \pm 0.01_9$	$0.00_0 \pm 0.00_0$
30	$0.01_1 \pm 0.01_2$	$0.00_0 \pm 0.00_0$	$0.01_9 \pm 0.03_0$
30 + 10^{-5} M eserine	$0.00_0 \pm 0.00_0$	$0.00_0 \pm 0.00_0$	$0.00_0 \pm 0.00_0$

Incubation mixtures contained the indicated substrate concentrations in μMoles/ml and 0.1 mg purified horse serum cholinesterase (Sigma) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Incubation was carried out at 37°C for the time indicated and controls for non-enzymatic hydrolysis in the presence of heat-inactivated enzyme were carried out for each incubation time and appropriate corrections made. The values represent the means of 8 determinations of ester hydrolysis in μMoles hydrolyzed ± standard deviation.

TABLE XIX

Hydrolysis of Acetyl-dl-carnitylcholine Chloride by Acetylcholinesterase

Substrate conc. / Time minutes	0.50	1.00	1.50 (µMoles/ml)	2.00	3.00
	µMoles hydrolyzed ± S.D.				
0	$0.00_0 \pm 0.00_0$	$0.00_5 \pm 0.00_0$	$0.00_6 \pm 0.00_0$	$0.01_0 \pm 0.01_0$	$0.03_9 \pm 0.02_5$
5		$0.17_3 \pm 0.04_0$	$0.21_7 \pm 0.01_0$	$0.29_6 \pm 0.02_5$	$0.38_2 \pm 0.01_5$
10	$0.05_1 \pm 0.00_0$	$0.24_1 \pm 0.02_0$	$0.32_5 \pm 0.01_0$	$0.44_6 \pm 0.01_0$	$0.54_1 \pm 0.00_0$
15	$0.11_9 \pm 0.00_5$	$0.26_2 \pm 0.01_0$	$0.38_6 \pm 0.02_5$	$0.46_9 \pm 0.00_0$	$0.61_5 \pm 0.00_0$
20	$0.14_1 \pm 0.00_0$	$0.27_5 \pm 0.00_0$	$0.40_4 \pm 0.01_0$	$0.47_6 \pm 0.00_0$	$0.68_9 \pm 0.00_0$
25	$0.14_4 \pm 0.00_5$	$0.28_6 \pm 0.03_5$	$0.39_8 \pm 0.02_5$	$0.49_4 \pm 0.02_5$	$0.75_2 \pm 0.02_5$
30	$0.14_0 \pm 0.00_5$	$0.28_0 \pm 0.00_5$	$0.38_6 \pm 0.02_5$	$0.51_2 \pm 0.00_0$	$0.76_5 \pm 0.05_0$
30 + 10^{-5}M eserine	$0.00_0 \pm 0.00_0$	$0.00_0 \pm 0.00_5$	$0.00_4 \pm 0.00_1$	$0.00_0 \pm 0.05_0$	$0.00_1 \pm 0.00_0$

Incubation mixtures contained the indicated substrate concentrations in µMoles/ml and 0.1 mg bovine erythrocyte AChE (Sigma) in a total volume of 1.0 ml made up in 0.1M sodium phosphate buffer, pH 7.0. Incubation at 37°C for the indicated times was terminated by addition of the alkaline hydroxylamine colour reagents. The degree of hydrolysis in µMoles hydrolyzed was determined by the modified Hestrin method (118). Controls for non-enzymatic hydrolysis consisted of incubation in the presence of heat-inactivated enzyme and appropriate corrections made. The values represent the mean values of 5 experiments ± S.D.

TABLE XX

Effect of Enzyme Concentration on the Hydrolysis of Acetyl-dl-carnitylcholine Chloride by Bovine Erythrocyte Acetylcholinesterase

Enzyme conc. μg	Substrate conc. μMoles	Reaction velocity Δ μMoles/10 min
20	0.99	0.075 ± 0.012
20	1.70	0.138 ± 0.035
20	2.20	0.238 ± 0.017
40	0.99	0.141 ± 0.019
40	1.70	0.235 ± 0.015
40	2.20	0.379 ± 0.073
60	0.99	0.175 ± 0.009
60	1.70	0.282 ± 0.000
60	2.20	0.411 ± 0.050
80	0.99	0.189 ± 0.034
80	1.70	0.320 ± 0.056
80	2.20	0.478 ± 0.000
100	0.99	0.223 ± 0.009
100	1.70	0.405 ± 0.025
100	2.20	0.522 ± 0.022
120	0.99	0.214 ± 0.009
120	1.70	0.430 ± 0.025
120	2.20	0.528 ± 0.000

Incubation mixtures contained in a total volume of 1.0 ml 0.1M sodium phosphate buffer, pH 7.0, the specified substrate and enzyme concentrations. Incubation was carried out at 37°C for 10 minutes and the reaction terminated by addition of the alkaline hydroxylamine colour reagents. The amount of ester hydrolyzed was determined according to the modified Hestrin method (118). Controls for non-enzymatic hydrolysis consisted of incubation in the presence of heat-inactivated enzyme, incubated in a boiling water bath for 10 minutes and these values were corrected for. The values presented are the mean values for 5 experiments ± S.D.

TABLE XXI

Effect of Enzyme Concentration on Hydrolysis of Acetyl-dl-carnitylcholine Chloride by Bovine Erythrocyte Acetyl-cholinesterase (Sigma)

ACCh conc. µMole	Active Enzyme µg	Inact. Enzyme µg	O.D. K.U.	∆O.D./15 min K.U.	% Hydrolysis
1.0	-	20	175.0		
1.0	-	100	175.0	-	-
1.0	20	-	162.6	12.4	7.1
1.0	40	-	151.9	23.1	13.2
1.0	60	-	141.0	34.0	19.4
1.0	100	-	128.7	46.3	26.5
2.0	-	20	348.2		
2.0	-	100	348.1	0.1	0.0
2.0	20	-	329.1	19.1	5.5
2.0	40	-	303.8	44.4	12.7
2.0	60	-	288.2	60.0	17.2
2.0	100	-	266.1	82.1	23.6
3.0	-	20	516.4		
3.0	-	100	516.0	0.4	0.0
3.0	20	-	486.4	30.0	5.8
3.0	40	-	456.3	60.1	11.6
3.0	60	-	440.0	76.4	14.8
3.0	100	-	391.2	125.2	24.2

Incubation mixtures contained in a total volume of 1.0 ml 0.1M sodium phosphate buffer, pH 7.0, the specified substrate and enzyme concentrations. Incubation was carried out at 37°C for 10 min and the reaction terminated by addition of the alkaline hydroxylamine colour reagents. The amount of exter hydrolyzed was determined according to the modified Hestrin method (118). Controls for non-enzymatic hydrolysis consisted of incubation with heat-inactivated enzyme which had been heated in a boiling water bath for 10 min. The values presented are the mean values for 5 experiments.

TABLE XXII

The pH Dependence of Acetyl-dl-carnitylcholine Chloride Hydrolysis by Bovine Erythrocyte Acetylcholinesterase (Sigma)

pH	mean Initial vel. ΔO.D./min K.U.	mean Initial vel. ΔµMole/min	% Hydrolysis ± S.D. %
4.10	0.7	0.004	0.30 ± 0.21
6.27	7.9	0.046	3.45 ± 0.36
6.57	10.0	0.058	4.36 ± 0.13
6.98	10.8	0.062	4.66 ± 0.34
7.51	11.0	0.064	4.81 ± 0.28
8.20	11.3	0.066	4.96 ± 0.27
8.75	9.8	0.057	4.28 ± 0.56
9.52	7.6	0.044	3.30 ± 0.28
10.07	2.0	0.012	0.90 ± 0.41

Incubation mixtures contained 1.33 µMoles dl-ACCh in 0.1 ml distilled water, 0.1% AChE solution made up in 0.1M sodium phosphate buffer, pH 7.2, and 0.8 ml of the appropriate buffers to give a final incubation volume of 1.0 ml. Omission of the substrate from the complete mixture constituted the reaction blank. Controls for non-enzymatic hydrolysis were obtained by incubating in the presence of heat-inactivated enzyme. The reaction was terminated after incubation at 37°C for 2, 4, 6, and 8 min by addition of the alkaline hydroxylamine colour reagents and the mixtures analyzed by the modified Hestrin method (118). The initial velocities were determined and the mean values from three experiments at each pH are presented.

Legend to TABLES XXIII and XXIV

Purified bovine erythrocyte acetylcholinesterase (Sigma), 2.0 mg in M/15 sodium phosphate buffer (pH 7.2) in a total volume of 2.0 ml, was heated at the indicated temperature for 5 minutes, cooled, and 0.1 ml aliquots assayed for hydrolytic activity by the modified Hestrin method using 3.0 μMoles ACh Iodide or 2.0 μMoles dl-ACCh Chloride as substrate in a total volume of 1.0 ml. Enzymatic activity was determined at 1, 2, 5, and 8 min intervals at 37°C. The initial velocity of the reaction was determined in μMoles substrate hydrolyzed per min and the mean values from 5 experiments are presented.

TABLE XXIII

The Effect of Temperature on the Hydrolysis of Acetylcholine Iodide by Bovine Erythrocyte Acetylcholinesterase (Sigma)

Temperature °C	mean ΔK.U./min	ΔμMoles/min	logΔ μMoles/10 min	T °A	1/T
7.0	8.0	0.08_8	0.94_8	280.0	0.0035_7
11.0	9.5	0.10_5	1.02_2	284.0	0.0035_2
14.5	10.5	0.11_7	1.06_7	287.5	0.0034_8
21.0	12.0	0.13_3	1.12_5	294.0	0.0034_0
23.0	13.0	0.14_5	1.16_0	296.0	0.0033_8
25.5	14.8	0.16_4	1.21_6	298.5	0.0031_9
30.0	15.0	0.16_7	1.22_2	303.0	0.0033_0
32.0	16.7	0.18_6	1.26_9	305.0	0.0032_8
37.0	17.7	0.19_6	1.29_3	310.0	0.0032_3
38.0	17.8	0.19_8	1.29_6	311.0	0.0032_1
39.0	19.1	0.21_2	1.32_7	312.0	0.0032_0
41.0	20.4	0.22_7	1.35_7	314.0	0.0031_8
49.0	19.1	0.21_2	1.32_7	322.0	0.0031_0
52.0	20.5	0.22_8	1.425	325.0	0.0030_7
60.0	15.6	0.17_4	1.24_1	333.0	0.0029_9
62.0	12.2	0.13_5	1.13_2	335.0	0.0029_8
64.5	5.6	0.06_2	0.79_7	337.5	0.0029_6
70.0	0.0	0.000	0.000	343.0	0.0029_1

TABLE XXIV

The Effect of Temperature on the Hydrolysis of Acetyl-dl-carnitylcholine Chloride by Erythrocyte Acetylcholinesterase

Temperature °C	mean ΔK.U./min	ΔμMoles/min	logΔμMoles/10 min	T °A	1/T
7.0	7.8	0.08_7	0.09_4	280	0.0031_9
11.0	9.8	0.10_9	1.03_5	284	0.0035_2
14.5	10.5	0.11_7	1.06_7	287.5	0.0034_8
23.0	13.0	0.14_5	1.16_0	296	0.0033_8
28.0	14.5	0.16_1	1.20_7	301	0.0033_2
31.0	15.0	0.16_7	1.22_2	304	0.0032_9
36.0	17.0	0.18_9	1.27_6	309	0.0032_3
39.0	17.7	0.19_7	1.29_5	312	0.0032_1
40.0	18.3	0.20_3	1.30_8	313	0.0031_9
50.0	18.2	0.20_3	1.30_8	323	0.0030_9
56.0	12.6	0.14_1	1.14_8	329	0.0030_4
62.0	9.5	0.10_5	1.02_3	335	0.0029_8
70.0	0	0	0	343	0.0029_1

TABLE XXV

The Effect of Enzyme Concentration on the Hydrolysis of Acetylthiocholine Iodide by Acetylcholinesterase

Enzyme conc. mg	O.D.	ΔO.D./10 min
0.00	0.100	-
0.02	0.148	0.048
0.04	0.197	0.097
0.06	0.241	0.141
0.08	0.282	0.182
0.10	0.315	0.215
0.20	0.470	0.370
0.30	0.495	0.395
0.40	0.495	0.395

Incubation mixtures contained 1.5 x 10^{-3}M acetylthiocholine, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of the concentration of bovine erythrocyte AChE (Sigma) indicated above made up in 0.1M sodium phosphate buffer (pH 7.4). Incubation was carried out for 10 minutes at 37°C, and the reaction terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The velocity of the reaction in change in O.D. at 540 mμ per 10 min is presented. The mean values from 8 determinations are presented.

TABLE XXVI

Effect of Incubation Time on the Enzymatic Hydrolysis of Acetylthiocholine Iodide by Acetylcholinesterase (Sigma)

Time min	O.D.	ΔO.D.
0	0.104	-
2	0.156	0.052
4	0.192	0.088
6	0.247	0.143
8	0.289	0.185
10	0.319	0.215
15	0.415	0.311
20	0.486	0.372
30	0.509	0.405
40	0.515	0.411

Incubation mixtures contained 1.5×10^{-3}M AcTCh, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.10 mg bovine erythrocyte AChE (Sigma) made up in 0.1M sodium phosphate buffer (pH 7.4). After intervals indicated, the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined and the mean values of 4 experiments are presented.

TABLE XXVII

The Effect of Substrate Concentration on the Hydrolysis of Acetylthiocholine Iodide by Acetylcholinesterase

Substrate conc. S (μMoles)	1/S	Initial velocity v (ΔO.D./min)	1/v
0.03_7	22.66	0.013 ± 0.001	80.0
0.07_5	13.33	0.046 ± 0.002	21.76
0.15	6.66	0.063 ± 0.001	15.87
0.18_7	5.33	0.077 ± 0.000	12.98
0.22_5	4.44	0.092 ± 0.000	9.70
0.30	3.33	0.120 ± 0.001	8.33
0.375	2.66	0.160 ± 0.003	6.25
0.75	1.33	0.210 ± 0.003	4.76
1.50	0.67	0.250 ± 0.005	4.00
1.87_5	0.53	0.250 ± 0.006	4.00
3.00	0.33	0.260 ± 0.006	3.84
3.75	0.27	0.260 ± 0.005	3.84
6.00	0.17	0.270 ± 0.007	3.70
9.00	0.11	0.260 ± 0.005	3.84
15.00	0.06	0.250 ± 0.006	4.00
37.50	0.026	0.246 ± 0.005	4.07

Incubation mixtures contained the indicated substrate concentrations in μMoles, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.1 mg bovine erythrocyte AChE (Sigma) made up in 0.1 ml 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2, 4, 6, and 8 min., the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined. The mean values ± S.D. for 5 experiments are presented.

TABLE XXVIII

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Eserine Sulphate

Inhibitor conc. x 10^{-7}M	Substrate conc., S, μMoles							
	0.30		0.45		0.75		1.50	
i	v	1/v	v	1/v	v	1/v	v	1/v
0.00	0.006_7	149.2	0.009_3	107.5	0.012_7	78.7	0.016_0	62.5
0.05	0.006_0	166.6	0.007_5	133.3	0.010_8	92.6	0.015_4	64.2
0.10	0.005_3	187.5	0.006_8	147.0	-	-	0.013_8	72.4
0.20	0.004_4	227.2	0.006_2	161.2	0.009_0	111.0	0.013_2	75.7
0.30	0.004_0	250.0	0.005_4	185.1	0.008_0	125.0	0.012_6	79.3

Incubation mixtures contained the indicated substrate and inhibitor concentrations, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1 ml 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2, 4, 6, and 8 min., the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity, v, in ΔO.D./min was determined. The mean values of 4 experiments are presented.

TABLE XXIX

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Acetylcholine Iodide

Inhibitor conc. µMoles	Substrate conc., S, µMoles							
	1.50		3.75		7.50		15.00	
i	v	1/v	v	1/v	v	1/v	v	1/v
0.00	0.013_1	76.3	0.015_7	63.7	0.013_2	75.7	0.017_3	57.8
1.50	0.008_5	117.6	0.013_5	74.0	0.013_3	75.1	0.017_0	58.8
3.00			0.010_6	94.3	0.012_2	81.9		
7.50	0.003_2	312.5	0.006_6	151.5	0.009_0	111.1	0.013_8	72.4
15.0	0.001_8	555.5	0.004_0	250.0	0.005_1	196.0	0.010_3	99.0
30.0	0.000_7	1428.5			0.002_7	370.3	0.006_8	147.0

Incubation mixtures contained the indicated substrate and inhibitor concentrations, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2, 4, 6, and 8 min., the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined and the mean values of 5 experiments are presented.

TABLE XXX

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Acetyl-ß-methylcholine Bromide

Inhibitor conc. µMoles	Substrate concentration, S, µMoles							
	0.50		0.62		1.00		1.25	
i	v	1/v	v	1/v	v	1/v	v	1/v
0.0	0.007_0	142.8	0.007_6	131.6	0.011_4	87.7	0.012_7	78.7
3.5	0.002_0	500.0	0.002_5	400.0	0.003_8	263.1	0.005_4	185.1
7.5	0.001_0	1000.0	0.001_2	833.3	0.002_4	416.6	0.002_8	357.1
15.0	0.000_6	1666.7	0.000_7	1428.0	0.001_2	833.3	0.001_4	714.2

Incubation mixtures contained the indicated substrate and inhibitor concentrations, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2, 4, 6, and 8 min., the reaction was terminated by addition of 0,1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined and the mean values of 3 experiments are presented.

TABLE XXXI

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Propionylcholine Chloride

Inhibitor conc. µMoles i	Substrate concentration, S, µMoles							
	0.37_5		0.75		1.50		3.00	
	v	1/v	v	1/v	v	1/v	v	1/v
0.00	0.006_4	156.2	0.010_6	94.3	0.014_0	71.4	0.015_8	63.3
1.25	0.003_4	294.1	0.006_4	156.2	0.009_2	108.7	0.013_0	76.9
2.50	0.002_6	384.6	0.004_8	208.3	0.007_6	131.6	0.010_0	100.0
3.75	0.001_8	555.5	0.003_6	277.8	0.005_5	181.8	0.009_0	111.1
5.00	0.001_4	714.3	0.002_6	384.6	0.005_0	200.0	0.006_8	147.0
10.00	0.000_8	1250.0	0.001_6	625.0	0.002_8	357.1	0.004_2	238.1

Incubation mixtures contained the indicated substrate and inhibitor concentrations, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2,4,6, and 8 min, the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in Δ O.D./min was determined and the mean values of four experiments are presented.

TABLE XXXII

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Acetyl-dl-Carnitylcholine Chloride

Inhibitor conc. µMoles i	Substrate concentration, S, µMoles							
	0.33		0.58		1.50		3.00	
	v	1/v	v	1/v	v	1/v	v	1/v
0.75	0.005_2	192.3	0.007_3	136.9	0.010_8	92.5	0.014_7	67.9
2.50	0.003_3	303.0	0.004_9	204.0	0.009_4	106.3	0.011_7	85.4
6.0	0.001_3	769.2	0.002_3	434.7	0.004_7	212.7	0.008_9	112.3
10.0	0.000_8	1250.0	0.001_3	769.2	0.003_1	322.5	0.005_7	175.4

Incubation mixtures contained the indicated substrate and inhibitor concentrations, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2,4,6, and 8 min, the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ∆O.D./min was determined and the mean values of 5 experiments are presented.

TABLE XXXIII

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Succinyldicholine Chloride

Inhibitor conc. µMoles	Substrate concentration, S, µMoles							
	0.375		0.75		1.875		2.25	
i	v	1/v	v	1/v	v	1/v	v	1/v
0.0	0.007_0	142.8	0.009_6	104.2	0.011_3	88.5	0.015_0	66.7
0.5	0.005_4	185.2	0.008_8	113.6	0.011_8	84.7	0.013_2	75.8
1.0	0.005_0	200.0	0.007_8	128.2	0.010_0	100.0	0.011_2	89.3
2.0	0.004_4	227.3	0.005_8	172.4	0.008_0	125.0	0.010_2	98.0
3.0	0.003_6	277.8	0.005_0	200.0	0.007_0	142.8	0.008_2	121.9
4.0	0.003_0	333.3	0.004_2	238.1	0.006_3	158.6	0.007_2	138.9

Incubation mixtures contained the indicated substrate and inhibitor concentration, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2,4,6, and 8 min, the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined and the mean values of 3 experiments are presented.

TABLE XXXIV

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by l-Carnitylcholine Chloride

Inhibitor conc. μMoles i	Substrate concentrations, S, μMoles									
	1.0		1.5		2.5		3.0		10.0	
	v	1/v	v	1/v	v	1/v	v	1/v	v	1/v
0.00	0.009_0	111.1	0.010_0	100.0	0.012_0	83.3	0.012_8	78.1	0.013_7	72.9
2.34	0.007_6	131.6	0.009_2	108.7	0.010_0	100.0	0.010_6	94.3	0.013_0	76.9
4.68	0.007_0	142.8	0.008_0	125.0	0.009_0	111.1	0.009_8	102.0	0.012_3	81.3
7.02	0.006_2	161.3	0.007_0	142.8	0.008_2	121.9	0.009_0	111.1	0.010_3	97.1
9.36	0.005_2	192.3	0.006_8	147.1	0.007_2	138.9	0.008_0	125.0	0.009_7	103.1

Incubation mixtures contained the indicated substrate and inhibitor concentrations, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1 ml 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2,4,6, and 8 min, the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined. The mean values of 3 experiments are presented.

TABLE XXXV

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by d-Carnitylcholine Chloride

Inhibitor conc. µMoles i	Substrate concentration, S, µMoles							
	1.0		1.5		2.0		2.5	
	v	1/v	v	1/v	v	1/v	v	1/v
0.0	0.010_2	98.1	0.012_0	83.4	0.012_4	80.7	0.012_4	80.7
0.1	0.007_2	138.9	0.009_2	108.8	0.010_4	96.1	0.010_6	94.4
0.2	0.006_4	156.3	0.008_2	121.9	0.009_0	111.1	0.009_8	102.1
0.3	0.005_6	178.7	0.007_4	135.1	0.008_4	119.0	0.008_8	113.7
0.4	0.004_8	208.3	0.006_2	161.3	0.007_4	135.1	0.007_8	128.1

Incubation mixtures contained the indicated substrate and inhibitor concentration, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2,4,6, and 8 min, the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined and the mean values of 3 experiments are presented.

TABLE XXXVI

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide in Butyrylcholine Iodide

Inhibitor conc. μMoles	Substrate concentration, S, μMoles									
	0.187		0.375		0.75		0.94		1.50	
i	v	1/v	v	1/v	v	1/v	v	1/v	v	1/v
0.0	0.004_0	250.0	0.007_0	142.8	0.009_0	111.1	0.011_7	85.5	0.014_1	70.9
7.5	0.003_4	294.1	0.005_4	185.1	0.007_5	133.3	0.009_4	106.4	0.012_8	78.1
15.0	0.001_8	555.5	0.004_0	250.0	0.005_3	188.6	0.006_0	166.7	0.009_2	108.7
30.0	0.001_0	1000.0	0.002_2	454.0	0.003_3	303.0	0.004_5	222.2	0.006_0	166.7

Incubation mixtures contained the indicated substrate and inhibitor concentrations, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1 ml 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2,4,6, and 8 min, the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined and the mean values of 4 experiments are presented.

TABLE XXXVII

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Choline Chloride

Inhibitor conc. μMoles i	Substrate concentration, S, μMoles									
	1.0		1.5		2.0		3.0		15.0	
	v	1/v	v	1/v	v	1/v	v	1/v	v	1/v
0	0.012_4	80.6	0.014_6	68.5	0.014_8	67.5	0.016_3	61.4	0.013_6	73.5
20	0.006_5	151.9	0.009_0	111.1	0.010_2	98.4	0.012_4	80.5	0.012_6	79.3
40	0.004_2	238.0	0.005_8	172.4	0.007_2	139.6	0.009_9	100.9		
60	0.003_1	322.5	0.005_0	200.0	0.005_8	172.4	0.007_8	127.3	0.011_1	90.1
100	0.001_9	526.3	0.003_2	312.5	0.003_8	263.1	0.006_1	164.4	0.009_7	103.1

Incubation mixtures contained the indicated substrate and inhibitor concentrations, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1 ml 0.1M sodium phosphate buffer (pH 7.4). After intervals of 2,4,6, and 8 min, the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined and the mean values of 4 experiments are presented.

TABLE XXXVIII

Competitive Inhibition of Acetylcholinesterase Hydrolysis of Acetylthiocholine Iodide by Acetyl-dl-Carnitine Hydrochloride

Inhibitor conc. μMoles i	Substrate concentration, S, μMoles					
	0.25		0.37_5		0.62_5	
	v	1/v	v	1/v	v	1/v
0	0.005_4	185.2	0.007_6	131.6	0.010_0	100.0
25	0.005_2	192.3	0.007_0	142.9	0.009_4	106.4
50	0.005_0	200.0	0.006_6	151.5	0.009_0	111.1
100	0.004_6	217.4	0.006_0	166.7	0.008_5	117.6
125	0.004_2	238.1	0.005_6	178.6	0.008_0	125.0

Incubation mixtures contained the indicated substrate and inhibitor concentrations, 0.5 ml 0.125M Tris buffer (pH 7.4), 0.2 ml 3.5M NaCl, and distilled water to a total volume of 1.4 ml. After equilibration at 37°C, the reaction was initiated by addition of 0.05 mg bovine erythrocyte AChE (Sigma) made up in 0.1M sodium phosphate buffer ()pH 7.4). After intervals of 2,4,6, and 8 min, the reaction was terminated by addition of 0.1 ml 25% TCA. Duplicate aliquots of 0.2 ml were assayed for sulphydryl content by the method of Grunert and Phillips (480). The initial velocity in ΔO.D./min was determined and the mean values of 4 experiments are presented.

TABLE XXXIX

Standard Spectrophotometric Assay for Choline Dehydrogenase

Time min	O.D. Choline	O.D. Control	ΔO.D. Choline	ΔO.D. Control	ΔO.D. Choline − ΔO.D. Control
0.0	0.890	0.890			-
0.5	0.740	0.860	0.150	0.030	0.120
1.0	0.660	0.830	0.230	0.060	0.170
1.5	0.595	0.800	0.295	0.090	0.205
2.0	0.540	0.780	0.350	0.110	0.240
2.5	0.480	0.760	0.410	0.130	0.280
3.0	0.435	0.740	0.455	0.150	0.305
3.5	0.390	0.730	0.500	0.160	0.340
4.0	0.355	0.715	0.535	0.175	0.360
4.5	0.315	0.700	0.575	0.190	0.385
5.0	0.285	0.695	0.605	0.195	0.410

Incubation mixtures contained 1.0 ml Krebs-Ringer phosphate buffer, pH 6.8, 0.2 ml 2.0% choline chloride or distilled water for controls, 0.5 ml 10 mg% 2,6-dichlorophenolindophenol, and distilled water to a total volume of 2.2 ml. Optical density (O.D.) readings at 607 mμ were taken at 30 second intervals after addition of 1.0 ml mitochondrial suspension. The results of a typical experiment are presented.

TABLE XL

The Effect of Substrate Concentration on the Oxidation of Choline Chloride by Choline Dehydrogenase

Choline conc. mg	ΔO.D. Choline per min	Δ O.D. Control per min	(ΔO.D.$_{\text{Choline}}$ - ΔO.D.$_{\text{Control}}$) per min
0.4	0.128	0.094	0.034
0.4	0.151	0.114	0.037
0.8	0.134	0.089	0.045
0.8	0.162	0.115	0.047
1.2	0.166	0.104	0.062
1.2	0.180	0.117	0.063
1.6	0.169	0.104	0.065
1.6	0.178	0.114	0.064
3.0	0.183	0.101	0.082
3.0	0.191	0.112	0.079
4.0	0.180	0.102	0.078
4.0	0.196	0.114	0.082
5.0	0.185	0.105	0.080
5.0	0.201	0.117	0.084

Incubation mixtures contained 1.0 ml Krebs-Ringer phosphate buffer, pH 6.8, the indicated substrate concentration or distilled water for controls, 0.5 ml 10 mg% 2,6-dichlorophenolindophenol, and distilled water to a total volume of 2.2 ml. Optical density readings at 607 mμ were taken at 30 second intervals after addition of 1.0 ml mitochondrial suspension. The initial velocity of the reaction in difference in change in optical density (Δ O.D.$_{\text{Exptl.}}$ - Δ O.D.$_{\text{Control}}$/min) are recorded with different choline concentrations. The results with 2 different enzyme preparations are presented.

TABLE XLI

Oxidation of Choline and Acetyl-dl-Carnitine by Choline Dehydrogenase

Time	(a) O.D.	(b_1) O.D.	(b_2) O.D.	(c) O.D.	ΔO.D.		
min	control	AC	AC+choline	choline	(b_1-a)	(b_2-a)	(c-a)
0.5	0.810	0.800	0.810	0.750	0.010	0.000	0.060
1.0	0.790	0.775	0.785	0.685	0.015	0.005	0.105
1.5	0.770	0.757	0.765	0.635	0.013	0.005	0.135
2.0	0.750	0.745	0.750	0.590	0.005	0.000	0.160
2.5	0.745	0.735	0.740	0.550	0.010	0.005	0.195
3.0	0.735	0.730	0.735	0.515	0.005	0.000	0.220
3.5	0.730	0.722	0.725	0.475	0.008	0.005	0.255
4.0	0.725	0.715	0.720	0.447	0.010	0.005	0.278
4.5	0.717	0.710	0.710	0.415	0.007	0.007	0.302
5.0	0.710	0.705	0.705	0.387	0.005	0.005	0.323

Incubation mixtures contained 1.0 ml Krebs-Ringer phosphate buffer, pH 6.8, 0.2 ml substrate ((a) distilled water; (b_1) 2.0 mg dl-acetylcarnitine; (b_2) 2.0 mg dl-acetylcarnitine plus 1.0 mg choline; (c) 1 mg choline) 0.5 ml 10 mg% 2,6-dichlorophenolindophenol, and distilled water to a total volume of 2.2 ml. Optical density readings following addition of 1.0 ml mitochondrial suspension were recorded at 607 mμ. Enzymatic activity was calculated in changes in O.D. with time.