CHOLINESTERASES AND ANTICHOLINESTERASE AGENTS

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SUB-EDITOR

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Preface

Although the anticholinesterase (anti-ChE) agents have only limited applications in therapy, and from the viewpoint of practical significance they are more appropriately classified as toxic compounds or insecticides than as drugs, in their capacity of pharmacological tools they have few equals. The concept of neurohumoral transmission was originally established largely from experiments in which physostigmine, or eserine, was employed to protect acetylcholine (ACh), the transmitter of the cholinergic nerves, from rapid hydrolytic destruction by acetylcholinesterase (AChE) and other cholinesterases (ChE's). Since then, a great number of additional reversible and irreversible anti-ChE agents also have been indispensable in studies of synaptic and neuroeffector transmission, and of other physiological processes. At the same time, there is practically no other class of compounds for which a mechanism of pharmacological action can be described in such concrete biochemical and physiological terms. Consequently, it is not surprising that a huge literature has developed on these several closely interdependent topics. The assembling and proper correlation of this material for the present volume has taken the collaborative efforts of over two dozen investigators. It is believed that their contributions to this end will prove invaluable to future investigators in providing a ready, inclusive source of established information, in defining areas where further studies are indicated, and in preventing unnecessary duplication of past work. How well these aims have been accomplished will be for time and the reader to judge.

The volume is divided into four major sections. The first (I, Chapters 1 to 6) presents the biochemical and physiological background which is essential to an understanding of the primary mechanism of action of the anti-ChE agents. This includes the identification and distribution of ACh and other naturally occurring choline esters (Chapter 1), the known facts about the enzyme choline acetylase (ChAc), which catalyzes the final step in the synthesis of ACh (Chapter 2), and the current knowledge and hypotheses concerning the formation, storage, and liberation of ACh *in vivo* (Chapter 3). Chapter 4 presents a classification of the cholinesterases (ChE's) and the methods employed for their determination. The nomenclature and abbreviations used here are with few exceptions followed in the other chapters of the volume. The embryonic appearance and development of ChE's in various phyla are considered in relation to function in Chapter 5. Chapter 6 describes the cytological localizations of AChE and other ChE's throughout the body, and considers the possible functions of the enzymes and their known substrate, ACh, on the basis of these observations and pertinent data from physiological studies.

Section II is devoted to the chemistry of the anti-ChE agents. This includes the biochemical problems of the nature of the reactions between the various types of inhibitors and the enzymes (Chapter 7), and the pathways of metabolic degradation of the organophosphorus anti-ChE agents (Chapter 10), as well as the chemical classifications and relationships between structure and pharmacological actions of the reversible (Chapter 8) and organophosphorus (Chapter 9) anti-ChE agents.

The systematic pharmacology of the anti-ChE agents is covered in Section III. Here, the authors have attempted to distinguish as well as possible between effects

due to inhibition or inactivation of ChE's and those which are more reasonably attributable to other mechanisms. The first four Chapters (11 to 14) of this Section discuss actions at sites where it is generally acknowledged that cholinergic transmission occurs, *i.e.*, at autonomic effector sites (11), autonomic ganglia (12), the neuromuscular junction of skeletal muscle (13), and certain regions of the central nervous system (14). The hypothesis that ACh and AChE are involved directly in the propagation of conducted axonal impulses is presented in Chapter 15; evidence to the contrary is considered in Chapter 6. The remaining two chapters here deal with the actions of anti-ChE agents on insects and other invertebrates (16), and on growth and development (17).

The final section (IV) treats the toxicological and therapeutic aspects of the anti-ChE agents. The general toxicological evaluation and the specific neurotoxic actions of the organophosphorus anti-ChE agents are presented in Chapters 18 and 19, respectively. Chapter 20 describes the pharmacology of the various types of antagonists of anti-ChE agents, with the exception of the compounds which reactivate alkylphosphorylated AChE; the latter are discussed in Chapter 21. The current clinical application of these findings to the treatment of intoxication with anti-ChE agents is presented in Chapter 22. Finally, the therapeutic uses of anti-ChE agents in myasthenia gravis (Chapter 23) and glaucoma (Chapter 24) are considered.

The Editor takes great pleasure in expressing to his collaborators in the preparation of this volume his deepest appreciation of their contributions and of their cooperative spirit and forbearance in bringing them into final form. He is most grateful for the invaluable editorial and secretarial help he has received from Miss Cornelia Geesey, Mrs. Zaroug Kabakian, and Mrs. Marian Sullivan. For the accomplishment of this own investigative work, much of which is included in Chapter 6, he is deeply indebted to the stimulating participation of his past and present colleagues whose work is cited and acknowledged there. It is particularly appropriate to note the inspiration which he received from his early mentor, the late Dr. Jonas S. Friedenwald, whose enthusiasm, genius, and kindly guidance first interested him in the application of histochemistry to pharmacological problems.

Finally, it is obviously not an Editor's prerogative to offer personal dedication of the work produced by his collaborators. However, his own efforts in assembling, contributing to, and editing this volume are dedicated with deepest affection to Win, and to Peter, Billy, and Jonathan, who provided both constant inspiration and generous relinquishment of their rightful claims to the hours taken for its compilation.

Philadelphia. September, 1961 G. B. K.

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Contents

D'autre part, la présence d'acétylcholine parmi les composants actifs de l'ergot de seigle, qui vient d'être signalée par Ewins, permet de supposer que, dans l'organisme animal, qui renferme, côte à côte, la choline et les acides gras supérieurs, et même certains dérivés contenant à la fois ces deux sortes de substances (lécithines), on pourra rencontrer un jour les homologues de l'acétylcholine, soit comme des constituants normaux des humeurs, soit comme les produits de certains états pathologiques.

(FOURNEAU and PAGE, 1914)

Introduction

Although acetylcholine (ACh) has been shown beyond reasonable doubt to be the transmitter substance at certain cholinergic nerve endings, there are several facts which warn us against attributing a too exclusive role to this compound. First, by analogy with other transmitters (e.g., the catecholamines) the transmitter role is likely to be subserved by a group of related substances rather than by a single compound. Second, ACh occurs in non-nervous tissue and is so widely distributed in nature as to suggest a non-nervous function for it. Third, several other carboxylic esters of choline possessing related or contrasting pharmacological properties are known to occur in nature. Though so far their presence in nervous tissue has not been unequivocally demonstrated, this tissue can undoubtedly synthesize homologues of ACh *in vitro* (GARDINER and WHITTAKER 1954, FRONTALI 1958, BERRY and WHITTAKER 1959) and is well equipped, by its possession of two forms of cholinesterase (ChE), to destroy them rapidly. The possibility that ACh may not be the only transmitter substance at cholinergic nerve endings or that its function may be interfered with under pathological conditions by the appearance of similar compounds must be borne in mind when considering the mode of action of anti-cholinesterase (anti-ChE) agents and justifies the inclusion of a chapter on the identification of ACh and related esters in a monograph on this subject.

No one test is specific for ACh in the concentrations at which it is likely to be encountered in biological material. Pharmacological tests are the most sensitive and can be made specific if used in combination. One method is that of *parallel* or *differential assays*, introduced by CHANG and GADDUM (1933), in which the unknown is quantitatively compared with a known ester in a series of pharmacological test systems which differ in their responsiveness to different esters. If the known and unknown esters are identical, the results of the assays should also be identical. Technical difficulties arise from the presence of interfering substances in tissue extracts, the relatively large error in biological assays, and the possible

Table 1. *Naturally occurring choline esters*

Ester	Species	Organ or tissue	References	Method of identification	Remarks
		Esters of definite occurrence and known constitution			
Acetylcholine	rye ergot	extract	1	A	First identification as natural product; probably formed by bacteria in extract (1a)
	horse	spleen	2, 3	(2) A, (3) CbD	First isolation from mammalian tissue (2)
		small intestine	4	D	First use of parallel assay
	human	placenta	4, 5	(4) D, (5) CbD	
	cat	superior cervical ganglion (perfusate during nerve stimulation)	6	D	
	dog	stomach (perfusate during nerve stimulation)	7	D	
	ox	spleen	8, 3, 9, 10	(8) CaD, (3) CbD, (9) Ca, (10) CaCcD	First use of paper (8) and column (3) chromatography; this tissue also contains propionylcholine (q.v.)
		small intestine	5	CbD	
		blood	11	A	Much smaller amounts found by (12)
		brain	13, 5, 14, 15	(13) A, (5) CaCbD, (14) CaD, (15) ACaCcD	Formed *in vitro* (13). Tissue also stated to contain butyrylcholine (q.v.)
	sheep	small intestine	5	CbD	
	goat	brain	5	CbD	
		small intestine	5	CbD	
		abomasum	5	CbD	
		rumen	5	CbD	
		heart	5	CbD	
	rabbit	brain	5	CbD	
		small intestine	5	CbD	
		heart	5	CbD	
	Octopus vulgaris	nervous tissue	16	AD	First demonstration in nervous tissue and in marine organism
	Mytilus edulis	gill plates	17	CaD	
	Torpedo marmorata	electric organ	18	Ca	
	Thais lapillus	whole organism	19, 20	CbD	
	Urosalpinx cinereus	whole organism	19, 20	CbD	
	lobster	ventral nerve cord	20	CbD	
	Myxine glutinosa	heart	21	Ca	A second active component present, but not identified
	housefly	head	22	CaBCaCc	
	honey-bee	head	23	Ca	
		royal jelly	24	ACaCc	
		honey	25, 26	(25) ABCaD, (26) E	
	Vespa crabro	venom	26a	CaB	
	Pieris brassica	eggs	27	CbD	
	Arctia caja	silk gland	27a	CaD	

Table 1. (cont.)

Ester	Species	Organ or tissue	References	Method of identification	Remarks
	Capsella bursae pastoris	—	28	A	
	Artocarpus integra	seeds and leaves	29	D	
	potato	tuber	30	ABCaD	
	Urtica urens	nettle hairs	31	D	
	Viscum album	—	32	A	
	Crataegus sp.	berries and leaves	33	CaD	
	Lactobacillus plantarum	whole organism	34	A	Earlier references to A identification in rotting vegetables given by (34)
	Lactarius blennius	press juice	1a	D	Esters not present in 36 other species of fungus, yeast, or fresh ergot
	Trypanosoma rhodesiense	whole organism	34a	D	
Propionylcholine	ox	spleen	8, 3, 9, 10	(8) CaD, (3) BCbD, (9) Ca, (10)CaCcD	Not found in other tissues examined by (5). First identification of a naturally occurring, pharmacologically active homologue of acetylcholine (8)
Acrylylcholine (I, $R_1 = R_2 = H$)	*Buccinum undatum*	hypobranchial gland	35	BCaCb	
Senecioylcholine (I, $R_1 = R_2 = Me$)	*Thais floridana*	hypobranchial gland	36, 20, 37	(36, 20) BCaCb, (37) A	Pharmacology studied by (38, 39)
Sinapylcholine (sinapine) (I,	*Sinapis alba*	seeds	40, 41	A	No reports of pharmacological activity
$R_1 = 3:5$-dimethoxy-4-hydroxyphenyl,	*Draba nemorosa*	seeds	42	A	
$R_2 = H$)	rape	seeds	42a	A	
Urocanylcholine (murexine) (I,	*Murex trunculus*	hypobranchial gland	43	AB	First isolated as a chemically unidentified base by (44); pharmacology studied by (45—48, 38)
$R_1 = 4(5)$-imidazolyl-, $R_2 = H$)	*M. brandaris*		44	A	
	Tritonalia erinacea		44	A	
	Urosalpinx cinereus		19, 20	BCaCb	
	Thais lapillus		19, 20	BCaCb	
	M. fulvescens		20	BCaCb	
γ-Aminobutyrylcholine	dog, pig	brain	49, 50	(49) Ca, (50) ABCa	Similar pharmacologically to fraction A of Factor I but not identical chromatographically (51). Has been studied pharmacologically by (52, 52a)
Esters of doubtful occurrence, unknown constitution or incompletely characterized					
Butyrylcholine	ox	brain	14	CaD	Found only in autolysing brain (15); can be synthesized by brain preparations *in vitro* (53) but acetylcholine only ester found in fresh brain (5, 15)

Table 1. (cont.)

Ester	Species	Organ or tissue	References	Method of identification	Remarks
Imidazolyl-acetylcholine	ox, horse, rat	brain	54	Ca	Results thought to be explained by presence of γ-aminobutyrylcholine (50)
Palmitylcholine	rat	liver	55	Cb	Synthesized *in vitro* by brain and liver (53)
Unidentified ester	honey-bee	head	23	Ca	
Unidentified ester	*Arctia caja*	cervical glands	56	Ca	Probably senecioylcholine
Unidentified ester	*Myxine glutinosa*	heart	57	Ca	
Unidentified ester	—	hypophysis	58	A	

Abbreviations: A, chemical; B, spectroscopic; Ca, by paper chromatography; Cb, by column chromatography; Cc, by paper electrophoresis; D, by parallel assay; E, enzymic; —, not stated.

References: (1) EWINS (1914), (1a) OURY and BACQ (1938), (2) DALE and DUDLEY (1929), (3) GARDINER and WHITTAKER (1954), (4) CHANG and GADDUM (1933), (5) KEYL (1957), (6) FELDBERG and GADDUM (1934), (7) DALE and FELDBERG (1934), (8) BANISTER et al. (1951, 1953), (9) AUGUSTINSSON (1955), (10) HENSCHLER (1957), (11) KAPFHAMMER and BISCHOFF (1930), (12) DUDLEY (1933), (13) STEDMAN and STEDMAN (1937), (14) HOLTZ and SCHÜMANN (1954), (15) HENSCHLER (1956b), (16) BACQ and MAZZA (1935), (17) BÜLBRING et al. (1953), (18) WOODIN, personal communication, (19) WHITTAKER and MICHAELSON (1954), (20) KEYL et al. (1957), (21) AUGUSTINSSON et al. (1955), (22) CHEFURKA and SMALLMAN (1956), (23) AUGUSTINSSON and GRAHN (1954), (24) HENSCHLER (1956a), (25) MARQUARDT and VOGG (1952a, b), (26) GOLDSCHMIDT and BURKERT (1955), (26a) BHOOLA et al. (1960), (27) DAVID (1959), (27a) MORLEY and SCHACHTER (1961), (28) BORUTTAU and CAPPENBERG (1921), (29) LIN (1955), (30) MARQUARDT, SCHUMACHER and VOGG (1952), (31) EMMELIN and FELDBERG (1947), (32) WINTERFELD (1942), (33) FIEDLER et al. (1953), (34) STEPHENSON and ROWATT (1947), (34a) BÜLBRING et al. (1949), (35) WHITTAKER (1959a), (36) WHITTAKER (1957), (37) WHITTAKER (1959b), (38) HOLMSTEDT and WHITTAKER (1958), (39) ERSPAMER and GLÄSSER (1958), (40) GADAMER (1897), (41) SPÄTH (1920), (42) KUNG and HUANG (1949), (42a) SCHWARZE (1949), (43) ERSPAMER and BENATI (1953), (44) ERSPAMER and DORDONI (1947), (45) ERSPAMER and GLÄSSER (1957), (46) TABACHNICK and ROTH (1957), (47) QUILLIAM (1957), (48) KEYL and WHITTAKER (1958), (49) KURIAKI et al. (1958), (50) KEWITZ (1959), (51) MCLENNAN (1959), (52) TAKAHASHI et al. (1958, 1959), (52a) HOLMSTEDT and SJÖQVIST (1960), (53) BERRY and WHITTAKER (1959), (54) GRUNER and KEWITZ (1955), (55) KENNEDY (1956), (56) BISSET et al. (1960), (57) AUGUSTINSSON et al. (1955), (58) FREUDENBERG and BILLER (1936).

existence of compounds which resemble each other in more than one test. Chromatographic methods, introduced by WHITTAKER and co-workers (WHITTAKER 1951b, WHITTAKER and WIJESUNDERA 1951, 1952a, BANISTER, WHITTAKER and WIJESUNDERA 1951, 1953, GARDINER and WHITTAKER 1954), have in recent years proved a valuable adjunct to purely pharmacological tests. If the compound in question fails to be identified with any known ester, identification can be achieved only by chemical methods which require the isolation of the active substance in milligram quantities or less.

The carboxylic esters of choline which are known or suspected in biological material are listed in Table 1. Some of them, e.g., propionylcholine, are intensely active substances: others, e.g., palmitylcholine, sinapine, are of doubtful physiological or pharmacological significance in the present context.

The ACh content of a very large number of different biological materials has been determined, usually by non-specific methods. In Table 1 the entries under ACh refer only to work in which at least some attempt was made to identify the active substance as ACh by parallel assay, chromatography, classical chemical, or physico-chemical techniques or a combination of these methods, and are intended to serve as a guide for future work of this kind. The relatively few

materials to which these more rigorous methods have been applied and the wide range of different phyla which have been found to contain ACh will both be apparent. The high concentration of ACh in certain plants is noteworthy, but perhaps not surprising when we consider its close chemical relation to the plant alkaloids, particularly esters of straight chain or ali-cyclic hydroxyalkylamines like aconitine and atropine. It is perhaps more surprising that no other carboxylic ester of choline has been isolated from plant materials with the exception of sinapine (reviewed by KAHANE and LÉVY 1938), which has not been reported to be pharmacologically active. This compound, like the snail esters (q.v.) is a β-substituted acrylylcholine, and is historically interesting as the oldest known derivative of choline; the latter was prepared from it and called sincaline by BABO and HIRSCHBRUNN (1852), the discoverers of sinapine, ten years before STRECKER (1862) invented the name "choline".

Between 1930 and 1933, KAPFHAMMER and co-workers (KAPFHAMMER and BISCHOFF 1930, BISCHOFF, GRAB and KAPFHAMMER 1932), using apparently unexceptionable chemical methods, reported the isolation of ACh from a number of tissues in much larger amounts than could be confirmed by other workers (for a recent study see MARQUARDT and HIRSCH 1952); for this reason, most of this work has been omitted from Table 1. DUDLEY (1933) made a careful attempt to repeat part of the work in KAPFHAMMER's own laboratory and reported an "uncanny" sudden increase in the ACh content of an extract during the isolation procedure. To the author's knowledge, a full account of this curious episode in the history of ACh research has never been published, though many of the details must be known to persons still living. Also omitted from Table 1 is the identification of ACh in milk (*cf.* ALM and AUGUSTINSSON 1957), which could not be confirmed by WHITTAKER (1958).

The possibility that homologues of ACh might occur in animal tissues was envisaged soon after EWINS' (1914) pioneer isolation of ACh from extract of ergot; as is shown by the quotation at the head of this chapter. However, it was not until 1951 that FOURNEAU and PAGE's prediction was realised by the isolation of propionylcholine from ox spleen by BANISTER et al. (1951, 1953). Although a fairly extensive survey carried out in the author's laboratory by KEYL in 1953—1954 and so far reported only in thesis form (KEYL 1957) failed to reveal the presence of propionylcholine in other mammalian tissues, the work of BANISTER et al. has stimulated interest in the whole question of the natural occurrence of other pharmacologically active esters of choline, and several more have now been identified in animal tissues. These include acrylylcholine (cation as in I, $R_1 = R_2 = H$) and two β-substituted acrylylcholines,

I

β,β-dimethylacrylylcholine (senecioylcholine) (I, $R_1 = R_2 = CH_3$) and β-imidazolyl-4(5)-acrylylcholine (urocanylcholine, murexine) (I, $R_1 = H$, $R_2 = C_3N_2H_3$), all present in the hypobranchial glands of different species of marine prosobranch gastropods of the division Rachiglossa, and γ-aminobutyrylcholine, present in mammalian brain.

Urocanylcholine was first isolated as far back as 1947 as a tissue base of unknown constitution by ERSPAMER and DORDONI, whose work is an excellent example of the value of parallel assays. They showed that the high ACh equivalence (for definition see section A. II p. 10) of the hypobranchial glands of species of the dye-secreting Muricidae, previously noted by VINCENT and JULLIEN (1938), could not be due to ACh because the pharmacological behaviour of the extracts differed from the latter in a series of tests. They succeeded in isolating the active substance as the crystalline styphnate and dipicrate and named it

murexine. The compound was identified chemically by ERSPAMER and BENATI (1953), synthesized by PASINI, VERCELLONE and ERSPAMER (1958) and shown to have the *trans* configuration by PASINI and VERCELLONE (1955b). Murexine is present also in three other Muricidae not examined by ERSPAMER and co-workers, *Urosalpinx cinereus*, *Thais lapillus* and *Murex fulvescens* (WHITTAKER and MICHAELSON, 1954), but the related *Thais floridana* contains senecioylcholine (WHITTAKER 1957, 1959b, KEYL, MICHAELSON and WHITTAKER 1957); *Buccinum undatum*, a member of the same order but not of the same family and not a dye-secreting snail, contains acrylylcholine (WHITTAKER 1959a). The salivary gland of another of the Buccinidae, *Neptunea antiqua*, contains a high concentration of neurine (FÄNGE 1958, EMMELIN and FÄNGE 1958); the hypobranchial gland of this species has not been examined. Senecioylcholine has also been identified with fair certainty in the cervical glands of the moth, *Arctia caja* (BISSET et al. 1960).

The biogenesis and the function of these esters are alike obscure. The acid moieties have carbon skeletons identical with those of the amino-acids histidine (urocanylcholine), valine (senecioylcholine), and alanine (acrylylcholine) and could be derived from them by α-deamination; *Thais lapillus* has, indeed, been found to be a fairly rich source of histidine α-deaminase (KEYL et al. 1957). The esters are present in the snail glands in high concentration; urocanylcholine and senecioylcholine possess neuromuscular blocking action of the depolarizing variety (ERSPAMER and GLÄSSER 1957, 1958, KEYL and WHITTAKER 1958, HOLMSTEDT and WHITTAKER 1958), a property which could account both for the high toxicity and the paralysing action of glandular extracts noted by DUBOIS (1909) who regarded the glands as venom organs. Secretion of senecioylcholine in *Arctia caja* likewise appears as part of a defence reaction which includes a threatening display (BISSET et al. 1960). By contrast, acrylylcholine has only an extremely weak blocking action, and is intermediate in properties between ACh and propionylcholine in a number of test systems (HOLMSTEDT, SUNDWALL and WHITTAKER unpublished). This raises the question as to whether the hypobranchial esters are paralysing toxins in all species. Invertebrate pharmacology and toxicology is still largely an unexplored field, and further work with the techniques now available might well bring to light many more choline esters.

The presence of γ-aminobutyrylcholine in brain, reported on somewhat slender evidence by KURIAKI et al. (1958), has been confirmed by KEWITZ (1959). There has been much discussion as to the relation of this ester and its parent acid to the inhibitory transmitter substance(s) assumed to be involved in central inhibition in the brain and spinal cord, and to Factor I, an inhibitory substance identified in brain by FLOREY and co-workers (FLOREY 1954, ELLIOTT and FLOREY 1956). According to the Japanese workers (KURIAKI et al. 1958, TAKAHASHI et al. 1958, TAKAHASHI et al. 1959), γ-aminobutyrylcholine has an intensely inhibitory action on the electrical activity of the cortex, about a thousand times more than γ-aminobutyric acid, but has otherwise little pharmacological activity of any kind. MCLENNAN (1959) and HONOR and MCLENNAN (1960) found that the ester resembles fraction A of Factor I in some respects but were unable to confirm its inhibitory action on cortical potentials. HOLMSTEDT and SJÖQVIST (1960) reported that it is largely inactive in a variety of peripheral test systems and is less active in some tests than the Japanese workers claimed. According to CURTIS, PHILLIS and WATKINS (1960), it resembles the free acid in depressing the ACh, glutamate, and synaptically evoked spikes of Renshaw cells, but differs from it in exerting no action on other spinal neurones. The depressant action on Renshaw cells is often followed by a prolonged excitation which appears to be an intrinsic property of the ester. However, the discovery of γ-aminobutyrylcholine undoubtedly opens up a

Table 2. *Methods available for the pharmacological assay of acetylcholine (ACh) and other naturally occurring choline esters*

The sensitivity is defined by the approximate threshold dose of ACh in $\mu\mu$mole/ml bathing fluid (isolated organs) or kg body weight (animal preparation). The numbers in brackets refer to the references at the foot of the table.

Method	Sensitivity	Specificity		Remarks
		Choline esters	Interfering substances	
Frog rectus abdominus muscle (1, 2)	25	Responds to large numbers of esters some of which are more active than ACh	Choline, potassium, phosphate, and phosphate esters give contractions but only in high concentrations. They may also potentiate the response to ACh but this can be allowed for	Requires only simple equipment. Muscle contracts reproducibly and relaxes rapidly, permitting speedy, accurate assays. Sensitivity of 5 μ μmole/ml attainable under favorable conditions with selected muscles
Guinea pig ileum (2)	10	Fairly specific for ACh	Histamine, substance P, 5-hydroxytryptamine also give contractions. Histamine response can be blocked by antihistamines	Requires more elaborate equipment than frog rectus. More likely to be interfered with by other biological components. Muscle contracts and relaxes rapidly but response is not so reproducible as frog rectus
Cat blood pressure (2)	5 to 10	Low specificity	Tissue vasoactive substances	Simple, rapid method. More expensive in animals than above methods. The rat (3) can also be used
Venus heart (4, 5)	0.5	Highly specific for ACh	Tissue vasoactive substances	Extremely sensitive and discriminating, but test organ not generally available.
Leech dorsal muscle (2, 6)	5	Specificity similar to frog rectus		Contracts less reproducibly than frog rectus. Relaxes very slowly. Assays more time consuming and less accurate than frog rectus
Buccinum undatum radula muscle (7, 8)	50	Specificity not known	Neurine gives ACh-like contraction. Tryptamine or 5-hydroxytryptamine added with ACh cause rhythmical contraction	
Frog lung (9)	5×10^{-7}			
Stichopus regalis dorsal muscle (10)	0.05	Specificity not known	Relatively unaffected by pressor amines	
Cat sciatic-gastrocnemicus (11)	—	Assays esters (e.g., urocanylcholine) having neuromuscular blocking action		
Crayfish stretch receptor (12)	—	Assays γ-aminobutyrylcholine and other inhibitory substances	Also responds to γ-aminobutyric acid	
Sea urchin oesophagus (13, 14)	—	Assays γ-aminobutyrylcholine and other inhibitory substances, e.g., fraction A of Factor I		Unaffected by γ-aminobutyric acid

(1) Chang and Gaddum (1933), (2) MacIntosh and Perry (1950), (3) Straughan (1958), (4) Wait (1943), (5) Welsh and Taub (1948), (6) Minz (1932), (7) Fänge (1958), (8) Fänge and Mattisson (1958), (9) Corsten (1940), (10) Bacq (1939), (11) Burn, Finney and Goodwin (1950), (12) Florey (1954), (13) Florey and McLennan (1959), (14) McLennan (1959).

new field for investigation in the pharmacology and biochemistry of choline esters; if it, or a related ester, turns out to be the much sought inhibitory transmitter, many facts concerning the specificity of the ChE's and choline acylases could be profitably re-examined.

The status of the remaining esters in Table 1 is less certain. A careful attempt to duplicate the work of HOLTZ and SCHÜMANN (1954) by KEYL (1957) failed to substantiate their claim to have identified butyrylcholine in ox brain; HENSCHLER (1956b) reported that this ester appears only in autolysing brain. The unidentified esters of bee brain (AUGUSTINSSON and GRAHN 1954) and *Myxine* heart (AUGUSTINSSON, FÄNGE, JOHNELS and ÖSTLUND 1955) might be chromatographic artifacts; this point is discussed in greater detail in Section B IV 2 c.

In the sections which follow, an account is first given of those properties of choline esters — pharmacological, chemical, spectroscopic and chromatographic — which are particularly useful for their identification and quantitative estimation. It is hoped that the fairly detailed treatment adopted will be useful not only to those working in the choline ester field but to all those who are interested in the identification of naturally occurring, pharmacologically active organic bases. With a few exceptions inserted for purposes of comparison, data are given only for known or suspected naturally occurring esters; for further information the reader is referred to the excellent monograph on biogenic amines by GUGGENHEIM (1951) and to the comprehensive survey of the pharmacology of synthetic drugs related to ACh by BOVET and BOVET-NITTI (1948). BARLOW's (1955) textbook also provides useful information. The chapter concludes with a section describing how the various properties of choline esters, as outlined in the previous sections, are utilized in their isolation and identification from biological material.

Properties of choline esters

A. Pharmacological properties

I. Introduction

It is not proposed to review in detail all the methods for the pharmacological assay of ACh as MACINTOSH and PERRY's (1950) account of the subject is still up-to-date. The main methods are summarized in Table 2. which indicates the sensitivity, specificity and general convenience of each. Also included are a few less well known methods the high sensitivity of which suggests that they would repay further investigation, and others which test properties possessed by some of the newer naturally occurring esters but not by ACh. It will suffice if in addition a few general points are mentioned concerning choice of materials and definition of terms.

No one method is specific for any one choline ester. This is not necessarily a disadvantage if it can be established by other means that the ester being assayed is the only one likely to be present or if the object is to determine the presence of unknown esters. More important than specificity are the stability of the preparation, the reproducibility and rapidity of its response, the degree of freedom from interference by adventitious substances, the simplicity of the equipment required, and the accessibility or cheapness of the test organism. By all these criteria the frog rectus assay method introduced by CHANG and GADDUM (1933) is probably the best. It is, however, somewhat less sensitive than other methods, and for the investigation of certain current problems there is a need for a really sensitive micro-assay method to complement the micro-pipette techniques which have been developed by the electrophysiologists. Possible methods include the

isolated frog lung (CORSTEN 1940) and various invertebrate preparations (BACQ 1947) such as the smooth muscle of the sea cucumber, *Stichopus regalis* (BACQ 1939), and the heart of the clam, *Venus mercenaria* (SMITH and LEVINE 1938, WAIT 1943, WELSH and TAUB 1948). The microbath of less than 50 μl capacity devised by GADDUM and STEPHENSON (1958) should prove useful in conjunction with some of these preparations; unfortunately, attempts to scale down conventional assay methods by using small slips of muscle run into the difficulty that the response tends to become all-or-nothing.

It is possible that purely electrophysiological methods of assaying choline esters will eventually be developed, using characteristic changes in electric potentials in the C.N.S. or endplate regions.

II. Definitions

As pointed out in section B II. 1 below, choline esters are stable in acid solution and unstable in alkaline; many, but not all, are also rapidly hydrolysed by ChE's. These properties serve to distinguish them from choline, potassium, phosphate, nucleotides and many other substances which are present in biological extracts and which may simulate or modify the effect of choline esters on the frog rectus and other preparations. Any assay of a biological extract should thus include a demonstration that the activity being measured is alkali-labile, and when the extract is being matched against the standard, an amount of alkali-treated extract equivalent to the dose of the extract should be added with the standard to ensure that the latter is exerting its effect against the same background as the unknown (FELDBERG and HEBB 1947). Assayed in this way the activity of an extract

Table 3. *Relative molar potencies of some choline esters*

Choline ester	Fall in blood pressure uneserinized cat	Contraction of eserinized frog rectus	Contraction of eserinized leech	Contraction of guinea-pig intestine	Neuromuscular blockade (*a*)	Crayfish stretch receptor (*b*)
Acetyl	100	100	100	100	—	—
Propionyl . . .	20 (1) 1 (2)	160—200 (3)	45 (4)	5—10 (3) 1 (5)	—	—
Butyryl[1] . . .	0.8 (1)	90—100 (3)	90 (4)	0.1 (5)	—	—
Palmityl . . .	—	—	—	0.1 (6)	—	—
Acrylyl[2]. . . .	10 (2)	30—90 (2, 7)	—	1—20 (2, 7)	0 (2)	—
Senecioyl . . .	rise (8)	20—70 (8, 9, 10*c*)	—	0.1 (8)	18 (8) 13 (10*c*)	—
Urocanyl[3] . . .	rise (8, 9, 10, 11)	10 (9, 11*c*)	—	0.02 (11*c*)	27 (8) 20 (11*c*)	—
γ-Aminobutyryl	0.1 (12)	0.025 (12*c*) 0.1 (13)	—	0 (12) 0.05 (13)	2.7 (13)	55 (14*cd*)

References: (1) SIMONART (1932), (2) HOLMSTEDT, SUNDWALL and WHITTAKER, unpublished, (3) BANISTER et al. (1953), (4) CHANG and GADDUM (1933), (5) SCHNEIDER and TIMMS (1957), (6) ABDERHALDEN, PAFFRATH and SICKEL (1925), (7) WHITTAKER (1959a), (8) HOLMSTEDT and WHITTAKER (1958), (9) KEYL et al. (1957), (10) ERSPAMER and GLÄSSER (1958), (11) ERSPAMER and GLÄSSER (1957), (12) KURIAKI et al. (1958), (13) HOLMSTEDT and SJÖQVIST (1960), (14) MCLENNAN (1959).

(*a*) Cat sciatic-gastrocnemius preparation (succinylcholine = 100). (*b*) γ-Aminobutyric acid = 100. (*c*) Calculated from authors' results, (*d*) on assumption that figures refer to free base.

[1] For higher homologues see BOVET and BOVET-NITTI (1948) and (5).

[2] For synthetic β-substituted acrylylcholines not included in table see (8).

[3] For synthetic heterocyclic choline esters not included in table see (10) and HOLMSTEDT, LARSSON and SUNDWALL (1960).