The Specificity of Pigeon Brain Aceto-cholinesterase*

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Earlier work by the author and coworkers (Adams & Whittaker, 1948; Adams, 1949; Whittaker, 1949; Mounter & Whittaker, 1950; Mounter, 1951) has shown that the so-called 'true' or 'specific' cholinesterases of erythrocytes, brain and cobra venom, previously believed to act only on acetylcholine and a few other esters, are in reality capable of hydrolysing a wide range of non-choline esters, 3:3-dimethylbutyl acetate,

$$(CH_3)_3C.CH_2.CH_2.O.CO.CH_3$$

the carbon analogue of acetylcholine,

$$(CH_3)_3N^+.CH_2.CH_2.O.CO.CH_3,$$

being the most rapidly hydrolysed of all non-choline aliphatic esters so far examined (for literature see Whittaker, 1951).

The work on brain cholinesterase has only been briefly reported (Whittaker, 1949). In this paper a fuller account of the specificity of pigeon-brain cholinesterase is presented, together with a few additional experiments on the localization of the enzyme in avian brain. Pigeon brain was selected on account of its high cholinesterase activity (nearly four times that of mammalian brain). It was shown by Adams & Thompson (1948) to resemble erythrocyte cholinesterase in its choline ester specificity. It has now been found to possess essentially the same pattern of specificity towards aliphatic esters as other aceto-cholinesterases (Sturge & Whittaker, 1950).

METHODS

Substrates and inhibitors. 3:3-Dimethylbutanol was prepared according to Birch (1949), with the following modifications. Ethyl cyano*iso*propylideneacetate was prepared according to Wideqvist (1949). Ethyl 2-cyano-3:3-dimethylbutyrate from the Grignard reaction was converted in one stage to *iso*propylmalonic acid by refluxing for 3 hr. with equal proportions by volume of H_2SO_4, acetic acid and water; after decarboxylation, the product was reduced to 3:3-dimethylbutanol with lithium aluminium hydride (Nystrom & Brown, 1947). Unsuccessful attempts were made to prepare the alcohol from di*iso*butylene (cf. Homeyer, Whitmore & Wallingford, 1933) and pinacone (cf. Cavalieri, Patterson & Carmack, 1945). The other substrates have been described by Sturge & Whittaker (1950).

Measurement of enzyme activity. This was performed manometrically, using Ammon's (1933) modification of the Warburg technique with the precautions and controls previously described (Sturge & Whittaker, 1950). 100 μmoles of aliphatic esters and 30 μmoles of acetyl-β-methylcholine (β-acetoxypropyltrimethylammonium chloride) were used per ml. Enzyme activity is expressed as amount of acid formed at s.t.p. in μl./mg. dry wt./hr. (Q value), in μl./ml./hr. or as a percentage of the acetyl-β-methylcholine (MCh) rate under identical conditions of pH, temperature and enzyme concentration.

Preparation of enzyme. Most tissues possessing cholinesterase activity contain one or more additional enzymes capable of hydrolysing aliphatic esters (ali-esterases) (Richter & Croft, 1942). Pigeon-brain homogenates were found to hydrolyse tributyrin and *n*-amyl butyrate at appreciable rates, substrates which are not appreciably attacked by the purified cholinesterase of red cells (Mendel & Rudney, 1943; Mounter & Whittaker, 1950). It was therefore suspected that pigeon brain also contained an ali-esterase, and attempts were made to free the cholinesterase from it. The course of the purification was followed by means of these two substrates and by *iso*amyl acetate which is split by both ali-esterase and cholinesterase.

The final method of purification was as follows. A pigeon was decapitated, the brain (including cerebellum) dissected out, the blood blotted off with filter paper, and the tissue homogenized for 2 min. in 20 ml. water in a Folley type homogenizer. A sample was withdrawn for testing (Q(MCh), 89). The remainder (15 ml.) was centrifuged on a bench angle centrifuge for 10 min. at 2000 g. The precipitate was resuspended in water (8 ml.) and again centrifuged. Only about 7% of the original activity now remained in the precipitate. The supernatant and washings were centrifuged at 16000 g. for 30 min. in an Ecco Ultima II centrifuge with stainless steel cups. The precipitate was resuspended in water (6 ml.) and again collected at 16000 g. for 30 min. This process was repeated. Yield, 34%; Q(MCh), 151. The product was stored at 0° in the presence of a drop of $CHCl_3$. Adsorption of this material on acid-washed kieselguhr (British Drug Houses Ltd.), at pH 5·5–6 and elution with aqueous 10 mM ammonia effected no further purification.

RESULTS

Table 1 gives the rate of hydrolysis of the four substrates by the untreated brain homogenate and the partially purified fraction. It will be seen that purification has eliminated the hydrolysis of the aliphatic butyrate shown by the original homogenate. By contrast, the hydrolysis of *iso*amyl acetate was reduced relative to acetyl-β-methylcholine, but not eliminated. This shows that the hydrolysis of the butyrates was due to an enzyme, distinct from the cholinesterase, which contributes

* For definition of 'aceto-cholinesterase' see Sturge & Whittaker (1950).

to, but does not completely account for, the hydrolysis of *iso*amyl acetate. This conclusion is supported by experiments (lower part of Table 1) with 10 μM-eserine. This concentration of eserine inhibits cholinesterases but not ali-esterases (Richter & Croft, 1942). Acetyl-β-methylcholine hydrolysis is 100 % inhibited in both preparations; the hydrolysis of the aliphatic butyrates is unaffected. The hydrolysis of *iso*amyl acetate by the purified fraction is abolished, but its hydrolysis by the original homogenate is not quite completely inhibited. The eserine-insensitive portion of the hydrolysis agrees well with the portion attributable to the ali-esterase in the unpurified homogenate. The results of Table 2 are consistent with competition of *n*- and *iso*-amyl acetate with acetyl-β-methylcholine for pigeon-brain cholinesterase. Although the purification procedure had effected but little concentration of cholinesterase activity, the primary object of the purification had thus been achieved in that the cholinesterase had been freed from ali-esterase activity. The specificity studies were therefore carried out with this preparation.

Table 1. *A comparison of unpurified and partially purified pigeon-brain cholinesterase*

	Untreated brain homogenate	Purified fraction
Q(MCh)	89	151
Rate of hydrolysis (% MCh rate)		
Tributyrin	18	<0·5
n-Amyl butyrate	4	1
*iso*Amyl acetate	71	53
Inhibition by 10 μM-eserine (%)		
Acetyl-β-methylcholine	100	100
*iso*Amyl acetate	76	100
Tributyrin	0	—
n-Amyl butyrate	0	—

Table 2. *Competition between aliphatic esters and acetyl-β-methylcholine for pigeon-brain cholinesterase*

Enzyme activity (μl./ml./hr.)			
Acetyl-β-methyl choline	*n*-Amyl acetate	*iso*-Amyl acetate	Mixed esters
832	264	—	764
1320	—	704	1160

Specificity. As far as choline ester specificity is concerned, pigeon-brain cholinesterase behaves like a typical aceto-cholinesterase (Fig. 1; Fig. 2, upper portion). Acetylcholine shows the phenomenon of inhibition by excess substrate; the optimum substrate concentration is about 3 mM. Acetyl-β-methylcholine and propionylcholine are also split, though less rapidly, and butyrylcholine is hardly

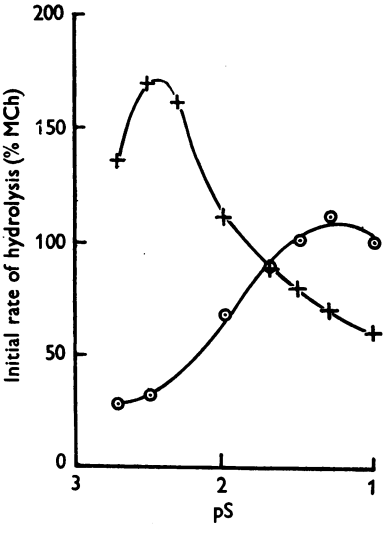

Fig. 1. Rate of hydrolysis of acetylcholine (×——×) and acetyl-β-methylcholine (⊙——⊙) as a function of substrate concentration. Ordinates: rate of hydrolysis as percentage of rate of hydrolysis of 30 mM-acetyl-β-methylcholine. Abscissae: negative logarithm of substrate concentration (pS).

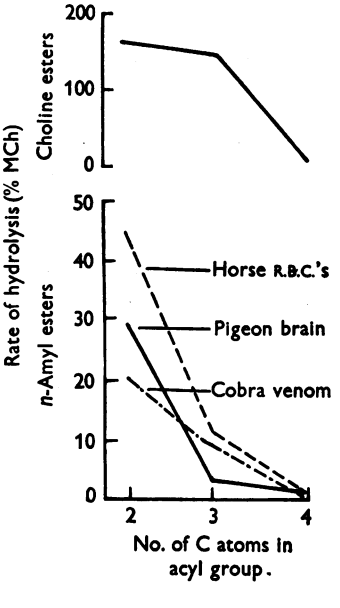

Fig. 2. Rate of hydrolysis of choline esters (upper portion) and *n*-amyl esters (lower portion) as a function of the size of the acyl group. Concentration of acetyl and propionylcholine, 3 mM; butyrylcholine, 10 mM.

attacked. Figs. 2 and 3 (continuous lines) show the relative rate of hydrolysis of a number of aliphatic esters by the pigeon-brain cholinesterase preparation. In Fig. 2 (lower portion) the rate of hydrolysis has been plotted against the number of carbon atoms in the acyl group of a series of *n*-amyl esters.

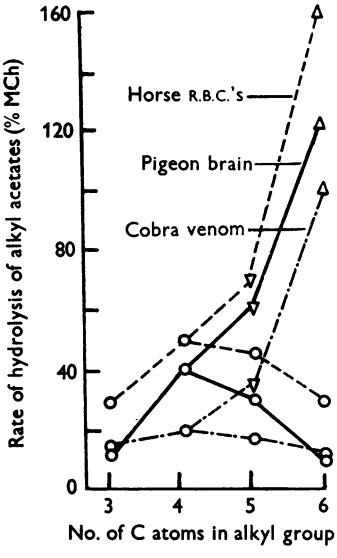

Fig. 3. Rate of hydrolysis of aliphatic acetates as a function of the size and configuration of the alkyl group. *n*-Alkyl acetates, ○; *iso*amyl acetate, △; 3:3-dimethylbutyl acetate, △.

As with the cholinesterases of horse erythrocytes (Mounter & Whittaker, 1950; broken lines) and cobra venom (Mounter, 1951; dotted and dashed lines) the rate of hydrolysis falls with increasing acyl-group size until with butyrate it is almost zero. A similar dependence on acyl-group size is seen with the choline esters (Fig. 2, upper portion). In Fig. 3 the effect of alkyl chain length and chain branching is presented. The abscissa now represents the number of carbon atoms in the alkyl group of a series of acetate esters; with the *n*-alkyl acetates the characteristic maximum at *n*-butyl acetate is once again observed as is the marked increase in rate on quaternization of the 3-carbon atom of *n*-butyl acetate. The specificity of pigeon-brain cholinesterase thus conforms precisely to the general pattern observed with the other aceto-cholinesterases.

Localization of avian-brain cholinesterase. During the purification of pigeon-brain cholinesterase, the impression was gained that the enzyme was attached to finely divided particulate material of varying particle size. The enzyme did not appear to be attached to grosser cell debris because, although much of it sedimented in centrifugal fields of low intensity (approx. 2000 *g*), washing these coarse particles or centrifugation of more dilute homogenates left relatively little activity in the precipitate. On the other hand, there was no evidence for two distinct 'fractions' of cholinesterase as described by Little (1948*a*, *b*) in mouse and dog brain, since higher speed centrifugation (16000 *g*) brought down appreciable proportions of the material in suspension at the lower speeds. It seemed likely that Little's 'supernatant' cholinesterase was merely a suspension of particles too fine to sediment in the ordinary laboratory centrifuge. To test this, dilute (2%) homogenates of mouse brain were prepared as described by Little (1948*a*) using water and 75 mM-sodium chloride. They were spun at 2000 *g* for 10 min. The distribution of activity in precipitate and supernantant was approximately as found by Little (Table 3). The supernatants were then spun at 16000 *g* for 30 min. There was some loss of activity in the water supernatant and considerable loss in the salt supernatant. Of the recovered activity, the greater part (72 and 77% respectively) was recovered in the precipitate. No doubt higher speeds and longer times would have led to even higher recoveries in this fraction. There is thus no evidence for separate forms of cholinesterase in brain, and the enzyme must be regarded as attached to particulate material as in the case of the red cell (Croft & Richter, 1943; Paléus, 1947; Augustinsson, 1948; Mounter & Whittaker, 1950). Probably salt concentration and other factors can

Table 3. *Distribution of mouse-brain cholinesterase between precipitate and supernatant of centrifuged homogenates*

(Figures in brackets are results of Little (1948*a*) inserted for comparison.)

Treatment	Percentage of recovered activity in: Precipitate	Supernatant	Activity lost (%)
2% Water homogenate:			
Homogenate centrifuged at 2000 *g*	5 (5)	95 (95)	0
Supernatant centrifuged at 16000 *g*	72	28	28
2% 75 mM-NaCl homogenate:			
Homogenate centrifuged at 2000 *g*	80 (65)	20 (45)	7
Supernatant centrifuged at 16000 *g*	77	23	—

influence the degree of aggregation of these particles as with the red cell preparations.

The localization of cholinesterase in mammalian brain has been the subject of several publications (Nachmansohn, 1939; Pighini, 1938; Birkhäuser, 1940; Augustinsson, 1948; Little, 1948*b*; Zeller, 1949; Burgen & Chipman, 1951; Ord & Thompson, 1952), but Nachmansohn's (1939) study of the domestic fowl appears to be the only systematic work with an avian brain. Nachmansohn found a considerably higher cholinesterase activity in the brain of this species than in mammalian brain, as the author has with the pigeon. Nachmansohn also showed that this high level of activity was a property of all parts of the brain, the optic lobes possessing a particularly high level of activity. This has been confirmed with the chick and is also true of the pigeon (Table 4). It is in marked contrast to mammalian brain, in which very varying levels of activity occur in different regions. Most authors have commented upon the high concentration of cholinesterase in the mammalian caudate nucleus and other basal ganglia, and the relatively low activity of the mammalian cortex. As the absolute activities given by different workers are somewhat difficult to compare, some figures for three mammalian species are included in Table 4 for comparison.

Table 4. *Cholinesterase activity of brains of various species*

(Determinations were carried out on pooled brains. Figures in brackets refer to the number of brains pooled.)

Avian brains		Mammalian brains	
Species	*Q*(MCh)	Species	*Q*(MCh)
Pigeon (4):		Cat (1)	22
Whole brain	87	Mouse (2)	30
Hemispheres	95	Rabbit (4):	
Optic lobes	147	Whole brain	26
Cerebellum	70	Caudate nucleus	105
Brain stem	43		
Chick* (6):			
Whole brain	88		
Hemispheres	86		
Optic lobes	143		
Cerebellum	72		
Brain stem	62		

* 5-day-old light Sussex cockerels.

DISCUSSION

The high level of avian-brain cholinesterase activity merits discussion. The phylogeny of the avian brain presents many difficulties, but it seems clear that the pallium of the primitive brain, from which the mammalian cortex is developed, is poorly represented in the avian telencephalon. The greater part of the forebrain represents a development of striatal tissue, from which the mammalian basal ganglia are also derived (cf. Kappers, Huber & Crosbie, 1936). The relatively higher cholinesterase content of the avian forebrain may thus be connected with the higher proportion of striatal tissue in this brain as compared with the mammalian brain. This does not, however, account for the high activity of the optic lobes, cerebellum and even the brain stem. No doubt the lower proportion of fibre tracts in the avian brain is an important factor (Nachmansohn, 1939). In the dog, the anterior lobe of the cerebellum, which may be more truly homologous with the avian cerebellum than the remaining portions of this organ, has a high activity, second only to the basal ganglia (Burgen & Chipman, 1951). The thalamus, corpora gemina and geniculate bodies, which in some degree must be regarded as homologous with the avian optic lobes, are also fairly active tissues.

The uneven distribution of the acetylcholine-aceto-cholinesterase-choline acetylase system in the various synaptic areas of the mammalian brain has led to the concept of two types of synaptic transmission in the central nervous system, cholinergic and non-cholinergic (Feldberg & Vogt, 1948). Possibly birds possess a greater proportion of the cholinergic synapses in their central nervous system, and a more detailed study of the localization of the components of the cholinergic system in avian brains might well contribute to the solution of this problem.

SUMMARY

1. Pigeon-brain cholinesterase has been shown to possess the specificity pattern characteristic of aceto-cholinesterases.

2. The high level of cholinesterase activity in the avian brain is confirmed and discussed.

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Gladiolic Acid, a Metabolic Product of *Penicillium gladioli*

2. STRUCTURE AND FUNGISTATIC ACTIVITY

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Gladiolic acid, an antifungal metabolic product of *Penicillium gladioli* Machacek has been shown to have the tautomeric structure I (Grove, 1952*a*; Raistrick & Ross, 1952). Ultraviolet and infrared spectroscopic investigation of gladiolic acid and its derivatives (Grove, 1952*b*) showed that whilst gladiolic acid was present in the lactol form I*a* (R = OH) in the solid state, an equilibrium between the lactol and open chain I*b* (R = OH) forms existed in aqueous solution, the pH being the determining factor. Furthermore, the ultraviolet-absorption spectrum of gladiolic acid in alkaline solution showed that the gladiolic acid anion had the hydrated (dihydroxyphthalan) structure II ($R = O^-$) although, as regards chemical activity, this structure was equivalent to I*b* ($R = O^-$); similarly, *o*-phthalaldehyde also existed in aqueous solution in the hydrated form (dihydroxyphthalan). In addition, the esters of gladiolic acid described by Grove (1952*a*) were shown to be pseudo esters of general formula I*a*.

Brian, Curtis & Hemming (1948) found that gladiolic acid inhibited germination of the spores of a number of fungi in Czapek-Dox medium at low pH, and Smith (1952) has presented data on the fungistatic activity of the related cyclopaldic and cyclopolic acids and on dihydrogladiolic acid. In the present paper the results of tests on a comprehensive series of derivatives of gladiolic acid and of simple model compounds chemically related to gladiolic acid are reported, and conclusions are drawn regarding the structural groupings which are responsible for the fungistatic properties of the gladiolic acid molecule. In addition, a number of other factors which influence the fungistatic activity of gladiolic acid are discussed in relation to the specificity shown by the antibiotic.

EXPERIMENTAL

Materials. The preparation and properties of the derivatives of gladiolic acid are described by Grove (1952*a*). Model compounds were prepared by standard methods and purified by distillation or crystallization.

Routine evaluation of fungistatic activity (Tables 1 and 2). Compounds were assayed by the spore-germination test with conidia of *Botrytis allii* Munn. (Brian & Hemming, 1945; Brian *et al.* 1948), but Czapek-Dox was used in place of Weindling medium throughout, since gladiolic acid is unstable in the latter. All solutions were adjusted to pH 3·5 before assay, and dilutions made in ×2 steps. Small discrepancies (within the limits of error arising from such a dilution procedure) between the figures quoted for the fungistatic concentrations of certain compounds in different sections of this paper are due to the dilution of solutions of differing initial concentration. Salicylanilide was used throughout as standard reference substance; all tests were carried out in duplicate and the mean figures quoted in the tables. The highest concentration tested was limited by the water solubility of the particular compound under investigation.