activity. Active urease preparations were obtained from all mouse stomachs examined.

4. None of thirty intact frog gastric mucosae showed any urease activity. The incubation media or secretions of resting or acid-secreting tied bags of mucosa contained no detectable quantities of ammonia, either in presence or absence of urea. Added urea was recovered quantitatively. Any acid-neutralizing role of urease in these experiments is thus excluded.

5. The amounts of acid secreted by isolated frog

gastric mucosa or whole mouse stomach in absence of added urea were more than thirty times the urea content of the tissue.

6. Since there is no evidence that urea is synthesized by gastric mucosa, it follows that urea and urease play no direct or catalytic role in the mechanism of acid secretion.

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The Specificity of Cobra-venom Cholinesterase

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The high concentration of cholinesterase in cobra venom was first noted by Jynegar, Sehra, Mukerji & Chopra (1938). The enzyme is present in the venom of the Colubridae, though absent from that of other genera, notably the Viperidae (Jynegar *et al.* 1938; Ghosh, Dutt & Chowdhury, 1939; Ghosh, 1940; Zeller, 1949). Following the recognition of two types of cholinesterase, Zeller & Maritz (1945) and Zeller (1947) classified cobra-venom cholinesterase as an *e*-type or 'true' cholinesterase.

Bovet-Nitti (1947a, b) studied the esterase specificity of cobra venom. She reported that a number of aliphatic esters such as the glycol and glycerol acetins and ethyl acetate were hydrolysed, but that tributyrin, ethyl propionate, amyl acetate and the choline esters of the higher fatty acids were not hydrolysed. The rate of hydrolysis of the *n*-alkyl acetates fell rapidly with increasing chain length. She assumed that the same enzyme was responsible for the hydrolysis of both acetylcholine

and the aliphatic acetates and, aware that this assumption did not conform to the views then current of the specificity of 'true' cholinesterases, proposed the name 'acetylase' for the enzyme (Bovet-Nitti, 1947*b*). Zeller (1948*a*) found a proportionality between the rate of hydrolysis of β -chloroethyl acetate, ethyl chloroacetate and the cholinesterase content of a number of snake venoms, and on this and other evidence concluded that the cholinesterase was capable of hydrolysing these aliphatic esters. He considered that the cholinesterases of colubrid venom constituted a separate class (ophiocholinesterases or *c*-type cholinesterases) with similarities to both types of mammalian cholinesterases (Zeller, 1948*a, b*). Following the first publication of Adams & Whittaker (1948), in which they reported the ability of purified human erythrocyte cholinesterase to hydrolyse a wide range of aliphatic acetate and propionate esters, and Holton's (1948) finding that cobra-venom cholinesterase resembled the *e*-type cholinesterases in hydrolysing triacetin, Bodansky (1946), Zeller & Utz (1949) and McNaughton & Zeller (1949) found that hydrolysis of the haloesters used earlier was not, in fact, a property characteristic of snake-venom cholinesterases, but was one shared also by typical *e*-type cholinesterases.

There remains, therefore, only one obstacle to classifying colubrid venom cholinesterases with those of brain and erythrocytes, namely the rapid fall in the rate of hydrolysis of *n*-alkyl acetates with increasing chain length reported by Bovet-Nitti, which contrasts with the rise up to *n*-butyl acetate followed by a fall found with brain and erythrocyte cholinesterases (Adams & Whittaker, 1948; Adams, 1949; Whittaker, 1949; Mounter & Whittaker, 1950).

The present work has been undertaken to determine whether, in fact, cobra-venom cholinesterase resembles in its aliphatic specificity the cholinesterases of brain and erythrocyte and whether it is to be regarded as a typical aceto-cholinesterase (Sturge & Whittaker, 1950).

METHODS

Measurement of enzymic activity. Rates of hydrolysis were measured by the Warburg manometric technique (Ammon, 1933) in which acid liberated by the hydrolysis of the substrate displaces an equivalent amount of CO_2 from a $\text{NaHCO}_3\text{-CO}_2$ buffer at pH 7.4 and 38°. Each flask contained a total volume of 3 ml., all solutions being made up in 0.023 M- NaHCO_3 , and after attachment to the manometers was gassed for 3 min. with 95% N_2 + 5% CO_2 . In most experiments the enzyme was placed in the side bulb and the substrate in the main compartment. The amount of enzyme was adjusted to give a gas evolution of 50–300 $\mu\text{l. CO}_2$ in 30 min. The enzyme was made up freshly for each experiment by dissolving dried venom in 0.023 M- NaHCO_3 , since it

was found that solutions of the enzyme deteriorated rapidly even at 0°. Non-enzymic controls were included in all experiments and the appropriate corrections made.

Source of enzyme. The cobra venom was kindly provided by Dr H. Blaschko and was part of the sample used by Blaschko & Stiven (1950). The species of cobra from which this venom was obtained is not known. It was collected in Madras in 1899, but despite the age of the sample it still retains a high cholinesterase activity, liberating 875 $\mu\text{l. CO}_2$ /mg. dried enzyme/hr. from 0.03 M-acetyl- β -methylcholine.

Substrates. Choline esters were obtained commercially, with the exception of propionylcholine, which was prepared in the laboratory as the perchlorate. The aliphatic esters were either commercial samples or were prepared in the laboratory and were all purified before use by washing, drying and distillation, the middle third of the distillate being collected. All esters satisfied the usual criteria of purity.

RESULTS

Hydrolysis of choline esters and water-soluble aliphatic esters

The pS initial rate curves were determined for acetylcholine, propionylcholine and acetyl- β -methylcholine. (pS is $-\log_{10}$ substrate concentration (M).) The results are shown in Fig. 1. The curves

Fig. 1. Effect of substrate concentration on rate of hydrolysis of various substrates by cobra-venom cholinesterase. isoAmyl acetate emulsified with gum acacia before use.

are similar in form to those obtained by Augustinsson (1949) with the enzyme of *Naja naja* venom. At a concentration of 0.03 M, acetyl- β -methylcholine (which has an almost flat-topped curve) gives a nearly maximal rate of hydrolysis, and this substrate at this concentration was used as a standard for subsequent experiments.

Triacetin at a concentration of 0.05M gave a similar rate of hydrolysis to that obtained by Blaschko & Holton (1949), who used a different sample of cobra venom (cf. Augustinsson, 1949). Both choline ester and triacetin hydrolysis are eserine sensitive (see below). Butyrylcholine is hydrolysed at a very low rate; benzoylcholine and tributyrin are not hydrolysed. The relative rates of hydrolysis of these substances are given in Table 1.

Table 1. *Initial rates of hydrolysis of various esters by cobra-venom cholinesterase*

Substrate	Concn. (M)	Rate of hydrolysis (as % of rate for acetyl- β -methylcholine)
Acetyl- β -methylcholine chloride	0.03	100
Acetylcholine chloride	0.006	240
Propionylcholine perchlorate	0.006	115
Butyrylcholine chloride	0.006	2
Benzoylcholine chloride	0.006	0
Triacetin	0.05	52
Tributyrin	0.05	0

Experiments with sparingly soluble aliphatic esters without added stabilizing agent

In the first experiments with aliphatic esters the procedure used in previous specificity studies (cf. Mounter & Whittaker, 1950) was adopted, i.e. esters were added directly to the flasks (300 μ mol./3 ml.) without previous emulsification, the flasks equilibrated for 10 min., the enzyme added from the side bulb and readings commenced after a further 5 min. Under these conditions it was found that ethyl and propyl acetates were hydrolysed, but that the acetates of higher alcohols were scarcely attacked (Table 2).

A possible explanation of these results was suggested by the work of Frazer & Stewart (1940), who found that oil-water emulsions of cobra venom were non-toxic when injected intravenously into rabbits. They attributed this to inactivation of the venom at the oil-water interface. The emulsion of sparingly soluble ester might have acted in the same way as the oily emulsions of Frazer & Stewart's experiments, bringing about a non-specific inactivation of the cholinesterase before it was able to attack the substrate appreciably. As it was not

known from Frazer & Stewart's experiments whether, in fact, inactivation of cholinesterase had occurred concomitantly with the loss of toxicity in their preparations of venom, some experiments were performed to test this hypothesis.

Table 2. *Rates of hydrolysis of aliphatic esters (300 μ mol./3 ml.) by cobra-venom cholinesterase expressed as percentage of acetyl- β -methylcholine rate: aliphatic esters present in part as separate phase*

Substrate	Rate
Ethyl acetate	6.5
Propyl acetate	4.6
Butyl acetate	2
Amyl acetate	1
isoAmyl acetate	2
Hexyl acetate	0
Amyl propionate	0

The inactivation of cobra-venom cholinesterase by oily emulsions and stabilization with gum acacia

A solution of cobra venom was shaken in a Mickie shaker for 5 min., alone or with paraffin, olive oil, diamyl ether or amyl butyrate (0.4 ml. of non-aqueous substance/10 ml. of venom solution); the last two substances were selected because of their chemical similarity to sparingly soluble substrates of aceto-cholinesterases. The cholinesterase activities of the samples were then compared with that of an untreated control using 0.03M-acetyl- β -methylcholine as substrate. Shaking for 5 min. was sufficient to produce a fine dispersion of the oily phase with formation of an extensive oil-water interface. Table 3 shows that in all samples containing an oily phase considerable inactivation had occurred and that inactivation was appreciable even when the enzyme was shaken in the absence of an oily phase. Experiments with a preparation of human erythrocytes, made by the method developed by Mounter & Whittaker (1950) for horse erythrocytes, are included for comparison. It will be seen that this cholinesterase, which hydrolyses sparingly soluble aliphatic esters without difficulty, is relatively stable under these conditions.

The most likely interpretation of these results is that advanced by Frazer & Stewart, namely, that the enzymes of cobra venom are (perhaps because of the relatively high concentration in which they

Table 3. *Inactivation of cholinesterases by shaking*

(Enzyme activities are percentage of unshaken control. Period of shaking, 5 min. in Mickie shaker, 0.4 ml. immiscible substance added/10 ml. enzyme solution.)

Source of cholinesterase	Shaken alone	Shaken with			
		Olive oil	Diamyl ether	Amyl butyrate	Paraffin
Cobra venom	48	20	15	12	38
—	—	6	8	3	—
Erythrocyte	98	65	61	62	92

occur in the dried crude venom) peculiarly sensitive to inactivation by adsorption into interfacial surfaces. It seems unlikely that the results were due to traces of inhibitors present in the substances added, as these were of the purest obtainable grade and unrelated chemically, though the possibility that some of these compounds are weak competitive inhibitors cannot be excluded as an additional factor. As a further test some experiments were carried out with emulsions of varying degrees of dispersion. Water-immiscible substances were added to the enzyme in the main compartment of the Warburg flasks, directly or after emulsification in the Mickle shaker. In the flasks to which unemulsified water-immiscible substances had been added some degree of emulsification was produced by the normal agitation of the flasks in the Warburg bath. Other flasks contained 5% (w/v) gum acacia with the object of bringing about increased dispersion.

Taking first the results obtained in the absence of gum acacia it will be seen (Table 4, col. 2) that with the exception of paraffin prior emulsification increased the inactivation of the enzyme by the various aqueous immiscible substances tested. The results are in line with the surface inactivation hypothesis; paraffin, which rapidly separated out into a separate layer, whether previously emulsified or not, gave the least inactivation; olive oil and amyl butyrate, which gave fairly stable emulsions, gave the greatest inactivation, while the reduction in rate resulting from the somewhat finer emulsions obtained from the Mickle shaker was greater than when the substances were added directly to the bottles.

The results obtained in the presence of gum acacia showed, however, an important difference. In each case the inhibitory effect of the oily phase was greatly reduced; the gum appeared to stabilize the enzyme in addition to stabilizing the emulsion. Possibly the gum itself is absorbed at the interface,

reducing the adsorption of the enzyme. Augustinsson's (1948) observation that gum acacia alone has a small potentiating effect on cholinesterases was incidentally confirmed (Table 4, cols. 2 and 3). The protection afforded is neither complete nor permanent; the rate of hydrolysis falls off more rapidly than does the control sample without added oil (Fig. 2).

Fig. 2. Effect of *n*-amyl butyrate on hydrolysis of acetyl- β -methylcholine.

In addition to gum acacia, gelatin and egg albumin were found to protect cobra-venom cholinesterase against the effect of oily emulsions. Sparingly soluble aliphatic esters are hydrolysed in the presence of these substances at about the same rate as in the presence of gum acacia (Table 5). Gum acacia proved, however, to be the most convenient stabilizing agent for general use as egg albumin was denatured after some minutes at 38°,

Table 4. *Inactivation of cobra-venom cholinesterase by water immiscible substances and its protection by gum acacia*

(Substrate, acetyl- β -methylcholine (0.03M); no. of experiments averaged shown in brackets.)

Aqueous immiscible substance added	Enzyme activity (% control without additions)		Protection afforded by gum acacia (%)
	Without gum acacia	With gum acacia (5%)	
None	100	109 (3)	—
Without previous emulsification:			
Olive oil	75 (2)	109 (2)	100
Amyl butyrate	42 (2)	96	88
Diamyl ether	95	100	92
Paraffin	90 (2)	—	—
With previous emulsification:			
Olive oil	9 (2)	68 (2)	63
Amyl butyrate	35	90	83
Diamyl ether	82	102	95
Paraffin	90	—	—

while gelatin was inconvenient to work with owing to the large change in viscosity between room and bath temperatures. All three protecting agents retarded somewhat the spontaneous loss of activity of venom cholinesterase solutions.

Table 5. *Hydrolysis of amyl acetates in presence of protecting substances*

(Rates expressed as percentage of rate with acetyl- β -methylcholine (0.03M); concentration of amyl esters, 150 μ mol./3 ml.)

Substrate	Gum acacia (5%)	Rate of hydrolysis in presence of	
		Egg albumin (5%)	Gelatin (2%)
isoAmyl acetate	40	53	43
n-Amyl acetate	25	33	28

Specificity studies with aliphatic esters in the presence of gum acacia

Specificity studies similar to those with other aceto-cholinesterases were now carried out in the presence of gum acacia, on the assumption that the

Fig. 3. Effect of acyl group size on hydrolysis of choline and aliphatic esters by cobra-venom cholinesterase.

gum would protect the enzyme against any non-specific inactivation by oily droplets of incompletely dissolved substrate, and that the rates of hydrolysis so obtained would give a truer picture of the specificity of the enzyme. The alternative, to work only with saturated aqueous solutions of aliphatic esters free from oily droplets, was rejected on account of the extremely low aqueous solubility, in the absence of the enzyme, of many of the esters which it was desired to investigate. As shown in Fig. 1, a total concentration of not less than about 150 μ mol./3 ml. is necessary to ensure the attainment of a maximum rate of hydrolysis of isoamyl

acetate, and an even higher concentration appears to be needed with triacetin (Augustinsson, 1949). The esters (150 μ mol.) were added to the flasks after having been first emulsified with 5% (w/v) gum acacia. (Addition of substrates to a mixture of gum and enzyme gave slightly lower rates.) The results are shown in Figs. 3 and 4.

It will be seen that the specificity pattern obtained is essentially the same as that of other aceto-cholinesterases (Adams, 1949; Whittaker, 1949; Mounter & Whittaker, 1950), the rate of hydrolysis of many esters which appear to be unattacked by the enzyme in the absence of gum acacia now being appreciable. Complete protection is probably not attained; thus the results were influenced somewhat by the amount of dispersion, and completely linear rates of hydrolysis were not obtained (Fig. 5). A

Fig. 4. Effect of size and configuration of alkyl group on hydrolysis of aliphatic esters by cobra-venom cholinesterase.

satisfactory degree of reproducibility was, however, ensured by means of the following precautions. Acetyl- β -methylcholine and later isoamyl acetate were included in all experiments as controls. The preparation of the emulsion was carefully standardized, the concentration of enzyme increased to give a relatively high rate of hydrolysis, and a lower ester concentration was used than in previous specificity studies. Although detailed substrate concentration-initial velocity studies were not carried out for every substrate, a few experiments with higher substrate concentrations suggested

that this concentration was enough to saturate the enzyme. Manometer readings were taken at 3 min. intervals, corrected for non-enzymic hydrolysis, and the extrapolated initial rate taken. No individual result differed from the mean to a sufficient extent to alter the specificity pattern.

Fig. 5. Time course of hydrolysis of isoamyl acetate (150 μmol./3 ml.) emulsified in gum acacia (5 %) by cobra-venom cholinesterase.

Evidence for absence of other carboxylic esterases

Ester hydrolysis was completely inhibited by 10 μM-eserine (Table 6), indicating that the hydrolysis was in fact due to cholinesterase and not to other carboxylic esterases (Richter & Croft, 1942).

Table 6. Inhibition of ester hydrolysis by eserine (10 μM)

Substrate	Enzyme activity (μ l. CO ₂ /ml./30 min.)		Inhibition (%)
	Control	Control + eserine	
Aqueous solutions of esters:			
Acetyl- β -methylcholine*	1470	0	100
Ethyl acetate†	80	0	100
Emulsions of esters in gum acacia:			
Butyl acetate†	450	0	100
<i>n</i> -Amyl acetate†	570	0	100
<i>iso</i> -Amyl acetate†	770	18	97
<i>iso</i> -Amyl propionate†	145	0	100

* 0.03 M.

† 150 μmol./3 ml.

DISCUSSION

The results presented above show that cobra-venom cholinesterase possesses the kinetics and specificity characteristic of aceto-cholinesterases. This extends to its behaviour towards aliphatic esters, the

apparent inability of the crude venom to hydrolyse many aliphatic esters which are attacked by other aceto-cholinesterases having been traced to a non-specific inactivation of the enzyme which is brought about by oily substances generally, irrespective of whether they are potential substrates or not. The rates of hydrolysis obtained in the presence of gum acacia are believed to represent the true rates of hydrolysis of these substrates, since: (a) gum acacia does not appreciably affect (actually slightly raises) the hydrolysis of a 'normal' substrate like acetyl-β-methylcholine (Table 4); (b) identical rates are obtained with diverse stabilizing agents (Table 5); and (c) the rate of hydrolysis of aliphatic esters by erythrocyte cholinesterase is unaffected by prior emulsification with gum acacia (unpublished results).

To summarize these characteristics (cf. Whittaker 1951) acetyl- and propionyl-choline are hydrolysed, giving humped Murray-Haldane curves similar to those obtained with erythrocyte, brain and electric-organ cholinesterase (Augustinsson, 1949); acetyl-β-methylcholine is also hydrolysed but not butyryl- or benzoyl-choline. With aliphatic esters there is an increase in the rate of hydrolysis of acetates and propionates as the choline configuration is approached, acetates being hydrolysed more rapidly than the corresponding propionates; no butyrate is hydrolysed. An exact comparison of the present results with those of Bovet-Nitti (1947a, b) cannot be made, but they do not appear to be incompatible if, as seems likely, Bovet-Nitti's experiments were carried out without the addition of a stabilizing agent or with aqueous solutions of esters. Certain experimental results of Zeller and his co-workers are also in agreement with those obtained here, although his earlier conclusion that cobra-venom cholinesterases comprise a separate class of enzyme, i.e. do not fall into either of the two groups of 'true' or 'pseudo' cholinesterase (otherwise aceto- and butyro-cholinesterase), is now seen to have arisen from the use of too few aliphatic substrates and from the lack of comparisons with other cholinesterases.

It has been previously pointed out that variations in details are to be expected in the specificity pattern of individuals within the main cholinesterase classes. Thus pigeon-brain cholinesterase hydrolyses aliphatic esters relative to acetyl-β-methylcholine at about one-half to three-quarters of the values obtained with erythrocyte cholinesterases (Whittaker, 1949). With the cobra-venom cholinesterase the values are still lower; moreover, there is a sharper fall with increase in size of the acyl group from acetate to propionate.

The conclusion that cobra-venom cholinesterase is a typical aceto-cholinesterase, differing only in detail from other members of this class is of special

interest for several reasons. It is the first cholinesterase of cold-blooded animals to be so identified; it occurs in a secretion, and not, as do other members of the class, in intimate association with structural elements of the cell; it is present in high activity in venoms whose neurotoxic properties are much in evidence. Much further work on this and the many other enzymes present in this secretion will, however, be necessary before a clear understanding of their biological significance is attained.

SUMMARY

1. The hydrolysis of choline and non-choline esters by cobra venom has been studied: all hydrolysis is attributed to cholinesterase.

2. It is suggested that the enzyme is inactivated

at interfaces giving an erroneous idea of the specificity pattern when sparingly soluble esters are used. The enzyme can, however, be protected by the addition of gum acacia or inert proteins.

3. The protected enzyme gives a specificity pattern similar to those found for other acetylcholinesterases. Acetates are hydrolysed faster than the corresponding propionates; butyrates are not hydrolysed. The rate of hydrolysis of aliphatic esters increases as the acetylcholine configuration is approached, giving a maximum with 3:3-dimethyl-butyl acetate.

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Observations on the Formation and Structure of Bacterial Cellulose

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Sisson & Clark (1933) and Sisson (1936), employing X-ray technique, studied cellulose produced from sugar by *Acetobacter xylinum*. Sisson's conclusions confirm and supplement earlier observations by Mark & Susich (1929), Hibbert & Barsha (1931) and Khouvine, Champetier & Sutra (1932). Later, bacterial cellulose produced from glucose by *A. xylinum* was examined by means of the electron

microscope by Franz & Schiebold (1943), while Frey-Wyssling & Mühlethaler (1946) applied the same technique to the study of cellulose formed by *A. xylinoides*. Mühlethaler (1949) has continued these studies using cellulose formed by *A. xylinum* in a beer-sucrose medium.

Kaushal & Walker (1947, 1951) reported the ability of certain species of *Acetobacter* other than