and penicillin production, except insofar as the disappearance of these fractions gave an indication of the general state of mould development. The output of penicillin by the mould followed the uptake of carbon, nitrogen and sulphur by the mycelium with a lag of 2-3 days. The reappearance of sulphur and nitrogen in the medium and the decline in mycelial carbon is probably related to senescence in the older parts of the mycelium, but further investigation is necessary. The peculiar fluctuations in phosphate content of the medium are as yet inexplicable. This phenomenon may be connected with the observation made by the author that the addition of phosphate buffer to growing cultures of *Penicillium notatum* produces only a transient change in the pH value which rapidly reverts to that of the parallel control culture. The inhibition experiment may be contrasted with results obtained by Mann (1943). This worker found that sodium iodoacetate ($M \times 10^{-3}$), sodium azide ($M \times 10^{-3}$) or sodium fluoride ($5M \times 10^{-3}$), when added to cultures of *Aspergillus niger* on medium containing 10% glucose and inorganic salts, caused a complete inhibition of the phosphorus metabolism and brought respiration to a standstill.

SUMMARY

1. The following metabolic fractions have been determined throughout the growth of *Penicillium notatum*: total carbon, reducing sugar, total nitrogen, amino-nitrogen, nitrate nitrogen, volatile base, phosphate phosphorus and total sulphur of the medium, total carbon and nitrogen of the mycelium, and the rate and quantity of CO_2 evolved by the growing culture.

2. Penicillin production by *P. notatum* followed the general absorption of carbon, nitrogen and sulphur into the mould mycelium.

3. No sharp discontinuities in the metabolism of *P. notatum* have been observed.

4. The exchange of phosphorus between the mould mycelium and the medium followed a fluctuating course which is as yet not understood.

5. The decline in the penicillin activity of the cultures was accelerated by the addition of ammonium ion although no pH change was produced by this treatment.

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Studies on the Specificity of Choline Esterase in *Helix pomatia*

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Since 1940 the problem of the specificity of cholinesterase has been placed in a new light, when it was shown by Alles & Hawes (1940) and Hawes & Alles (1941) that the enzymes in human blood serum and red blood cells had different properties. Among other things, these authors found that excess acetylcholine depressed the activity of the erythrocyte enzyme but not that of the serum enzyme. These observations were subsequently confirmed in

different quarters. According to Richter & Croft (1942) cholinesterase in the erythrocytes is different from that in the blood serum, being exclusively specific toward choline esters and being affected differently by certain substances. Similar results were also obtained by Mendel & Rudney (1943*a*), according to whom it is 'clear beyond doubt that two distinct cholinesterases exist in the animal body' (1944). Zeller & Bissegger (1943) found their

e-type in erythrocytes and brain, and their *s*-type in blood serum. The *e*-type is also found in skeletal muscle (Langemann, 1944; also Nachmansohn & Rothenberg, 1945). In certain blood sera and tissues a mixture of the two types has been obtained, and also, according to Glasson (1944), in red blood cells. A definite proof that two distinct cholinesterases really exist in red blood cells and serum has been provided by the present author (1944, 1945) in a cataphoretic investigation. Mendel & Rudney (1943*b*) found true cholinesterase only in brain tissues of vertebrates, but according to Nachmansohn & Rothenberg (1944, 1945) 'the esterase in all nerve tissues is either exclusively or predominantly' a specific cholinesterase; this seems also to hold for insect nerves (Richards & Cutkomp, 1945). A comparison between the two types of cholinesterase is found in Table 1, which is partly of interest in relation to the following discussion.

Table 1. *Comparison of the two types of acetylcholine-hydrolyzing enzymes*

Nomenclature	{ Unspecific pseudo <i>s</i> -Type	Specific true <i>e</i> -Type	Nachmansohn & Rothenberg (1945); Mendel & Rudney (1943 <i>a</i>); Zeller & Bissegger (1943)
Optimum pH	8.5	7.5-8.0	Alles & Hawes (1940)
Isoelectric point	4.35	4.65-4.70	Augustinsson (1944, 1945)
Stability at different pH values	More stable	Less stable	
Inhibition by excess of substrate	-	+	Alles & Hawes (1940); Mendel & Rudney (1943 <i>a</i>); Zeller & Bissegger (1943); Nachmansohn & Rothenberg (1945)
Activation by NaCl	-	+	Alles & Hawes (1940)
Hydrolysis of: Tributyrin	+	-	Vahlquist (1935); Richter & Croft (1942); Mendel & Rudney (1943 <i>a</i>); Nachmansohn & Rothenberg (1945)
Acetyl- β -methylcholine	-	+	Alles & Hawes (1940); Mendel & Rudney (1943 <i>a</i>); Nachmansohn & Rothenberg (1945)
Benzoylcholine	+	-	Mendel & Rudney (1943 <i>a</i>); Nachmansohn & Rothenberg (1945)

In order to establish whether this 'type theory' is applicable to different species of animals, the author has studied cholinesterase occurring in some primitive organisms. The present communication deals with the results obtained with *Helix pomatia* (cf. Augustinsson, 1946*b*). An investigation with some marine invertebrates is in press elsewhere (Augustinsson, 1946*a*); that paper also contains a review of earlier studies on the cholinesterase content of invertebrates.

METHODS

Measurement of activity. The cholinesterase activity was measured with the Warburg manometric method (Augustinsson, 1944). In the main compartment of the flask were placed 1.6 ml. of the substrate solution, and in the side bulb, 0.4 ml. of the enzyme solution. The hydrolysis was carried out at 37.5° in a gas mixture of 95% N₂ and 5% CO₂. The solutions, were saturated with this mixture and the flasks filled after attachment to the manometers. Readings

were made continuously for 60 min. after addition of the enzyme to the substrate solution.

Preparations of the substrate solutions. The substrates were dissolved in bicarbonate-Ringer solution (*R*₃₀) of the following composition:

Solution	% (w/v)	ml.
NaCl	0.90	100
NaHCO ₃	1.26	30
KCl	1.20	2
CaCl ₂ (cryst.)	1.76	2

For each experiment fresh *R*₃₀ was used. The substrates were: acetylcholine chloride (Hoffmann-La Roche), acetyl- β -methylcholine chloride (Merck), benzoylcholine chloride (Hoffmann-La Roche), acetylneurin chloride hydrochloride (Hoffmann-La Roche), tributyrin (Schering-Kahlbaum), ethyl acetate, and these names are abbreviated in the figures to ACh, MeCh, BzCh, AAn, TB and EA respectively.

Six solutions of the substrates were used with the following percentage concentrations (w/v): 2.5, 0.75, 0.25, 0.075, 0.025, 0.0075. In some cases, only the 0.25% (w/v) solution was used.

Enzyme preparations. Different tissues of *H. pomatia*, the edible snail, were used. The snails were collected in the early autumn just before the winter sleep, and kept in a box with soil and leaves. In this captivity the animals shortly made ready for hibernation in the usual manner. In the first months the author could find no changes of the cholinesterase activity in the blood and the dart sac of the snails. Other tissues were examined before the winter sleep commenced.

A hole was made in the shell and the blood drawn from the circulus venosus. The whole shell was removed and the body dissected. The following tissues were studied: the head with the central nervous system, the foot, the digestive gland ('liver'), the brown liquid secreted by the 'liver', the hermaphrodite gland (ovotestis), the mucous glands, the dart sac without the dart, and the blood.

Each tissue (5-10 g.) was ground in a mortar with washed sand and the disintegrated material taken up with 2 parts of *R*₃₀ solution. The mixture was shaken for some minutes, centrifuged at a constant speed of 3000 r.p.m., and the fluid decanted. The buffered suspensions were kept in the

refrigerator for 4 days, during which period the analyses were made. The preparations did not change in activity during this time.

In the experiments with horse blood, this was defibrinated by stirring immediately after withdrawal and then centrifuged. The blood cells were washed three times with 0.9% NaCl solution and then haemolyzed with distilled water. The brain preparations from dog were made in a way similar to that described above for the snail tissues.

RESULTS

Autolysis of substrates. In the first place, it was important to determine the degree of autolysis of the substrate solutions used. The results of such

Fig. 1. Autolysis of acetylcholine, acetylmethylcholine, benzoylcholine and acetylcholine as a function of concentration.

determinations are found in Fig. 1. In these experiments substrate solutions were mixed with R_{30} instead of an enzyme preparation. Table 2 gives the final substrate concentrations used in the investigations reported in this paper.

Enzyme activities of some tissues of *H. pomatia*. The determinations of the cholinesterase activities

of different tissues of the edible snail gave some interesting results. Table 3 gives the results of some typical experiments.

Table 2. Final concentrations of the substrates after mixing the enzyme solutions or R_{30} (autolysis) with substrate solutions

(Total volume in the main compartment 2.00 ml.,
pS = -log molar concentration.)

Solution no.	Acetylcholine, mol. wt. 181.7		Acetylmethylcholine, mol. wt. 195.7		Benzoylcholine, mol. wt. 243.6	
	M	pS	M	pS	M	pS
1	1.10×10^{-1}	0.96	1.02×10^{-1}	0.99	0.82×10^{-1}	1.09
2	3.30×10^{-2}	1.48	3.07×10^{-2}	1.51	2.46×10^{-2}	1.61
3	1.10×10^{-3}	1.96	1.02×10^{-3}	1.99	0.82×10^{-3}	2.09
4	3.30×10^{-3}	2.48	3.07×10^{-3}	2.51	2.46×10^{-3}	2.61
5	1.10×10^{-3}	2.96	1.02×10^{-3}	2.99	0.82×10^{-3}	3.09
6	3.30×10^{-4}	3.48	3.07×10^{-4}	3.51	2.46×10^{-4}	3.61
			4.08×10^{-1}	0.39		

In the hydrolysis of acetylcholine, the dart sac is surprisingly active. Nachmansohn's Q_{ChE} (mg. acetylcholine hydrolyzed by 100 mg. tissue in 60 min.) is about 10; in comparison, Q_{ChE} for the frog muscle is 0.4–0.6 (Marnay, 1937). As far as the author is aware the innervation of the dart sac is not known.

The snail blood also has a very high activity which is about four times higher than that of horse blood serum. Thus, in a comparative experiment with snail blood and horse serum, diluted to the same degree, the activities expressed in b_{30} (cf. Table 3) were 205 and 50 respectively. This confirms an early observation of Ammon (1935).

The following observations are also of interest. The activities in the blood and the dart sac are quite different. Especially noticeable are the high rate of hydrolysis of acetylcholine caused by the blood, and that of tributyrin by the dart sac. The same factor can scarcely be responsible for the two effects. The choline-ester hydrolyzing enzyme in the blood

Table 3. Enzymic hydrolysis of various esters by some snail tissues

(The activity is expressed as $b_{30} = \mu\text{l. CO}_2$ evolved during 30 min., and calculated for 100 mg. tissue. The substrate concentrations of acetylcholine, acetylmethylcholine, and benzoylcholine are those of the solutions No. 3 (Table 2). The others are: 0.0055 M-acetylcholine, 0.0066 M-tributyrin, 0.0227 M-ethyl acetate.)

	$b_{30}/100 \text{ mg. tissue}$					
	Acetylcholine	Acetylmethylcholine	Benzoylcholine	Acetylcholine	Tri- butyrin	Ethyl acetate
Head with C.N.S.	132	26	60	34	302	60
Foot muscle	148	40	65	35	109	12
Digestive gland ('liver')	90	52	33	25	133	80
'Liver' juice	122	18	46	—	—	—
Hermaphrodite gland	65	16	2	—	—	—
Mucous glands	127	6	10	—	—	—
Dart sac	620	38	18	34	164	0
Blood	760	239	11	196	85	5

may be a specific cholinesterase active toward acetyl-methylcholine. However, it is very probable that the hydrolysis of the latter and of acetyl-aneyrin is caused by the same factor. It is very improbable that this latter is the enzyme hydrolyzing acetylcholine (see below). The dart sac contains, in all likelihood, a lipase and a cholinesterase which has a degree of specificity toward acetylcholine formerly unknown.

The other tissues examined doubtless also contain a mixture of esterases. The 'liver' juice may contain an unspecific cholinesterase; a peculiar and as yet unexplained fact is that this enzyme is not inhibited by physostigmine (Režek & Haas, 1942). The same may be the case with the head and the foot muscle which very probably also contain lipases or other esterases. A specific cholinesterase in the 'liver' may split acetylcholine and acetyl-methylcholine; an unspecific esterase, benzoylcholine, acetyl-aneyrin and ethyl acetate a lipase, tributyrin. This is a working hypothesis which will form the basis of further studies.

Cholinesterase activity of the blood of H. pomatia compared with that of the brain and red blood cells from vertebrates. A closer study of the cholinesterase of *Helix* blood has given the following results. According to the preliminary investigation reported above, the enzyme in snail blood may be a specific cholinesterase, in agreement with the hypothesis of Mendel, Mundell & Rudney (1943) that acetyl- β -methylcholine is hydrolyzed by true cholinesterase only and not by the pseudo-enzyme, while benzoylcholine is hydrolyzed only by pseudo and not by true cholinesterase. The other main characteristic of a specific cholinesterase is the depressing effect on the hydrolysis caused by a substrate concentration above the optimum. This effect is found with the snail blood when acetylcholine is used as substrate (Figs. 2, 4). The optimum acetylcholine concentration is close to $3 \times 10^{-3}M$, which is somewhat less than that previously reported for the specific enzyme.

When acetyl-methylcholine is employed as substrate, such a depressing effect is not found, at least in the usual concentration range (Figs. 3, 4). It is very possible that the rate of hydrolysis decreases when the concentration of acetyl-methylcholine is higher than $0.5M$, which is the optimum. In such a case, the activity-pS curves for acetylcholine and acetyl-methylcholine are merely displaced in relation to each other. Such high substrate concentrations, however, are not natural to the cholinesterase system. The difference in the cholinesterase activity towards these two substrates appears also in the shapes of the hydrolysis curves. The hydrolysis of acetylcholine seems to be a zero-order reaction, that of acetyl-methylcholine a first-order reaction.

Fig. 2. Enzymic hydrolysis of acetylcholine by *Helix* blood diluted 1 to 9 with R_{30} . The numbers refer to the solutions in Table 2.

Benzoylcholine gives an S-shaped curve, similar to that of acetyl­methylcholine. This is also the case with acetyl­aneurin (Table 4).

For comparison, similar experiments have been performed with brain (dog) and red blood cells (horse). In these cases, excess of acetylcholine inhibits the hydrolysis, confirming earlier results by other workers (see Table 1). The present author has

Fig. 3. Enzymic hydrolysis of acetyl­methylcholine by *Helix* blood diluted 1 to 9 with R_{30} . The numbers refer to the solutions in Table 2.

Table 4. Enzymic hydrolysis of benzoylcholine and acetyl­aneurin by *Helix* blood, and of acetyl­methylcholine and benzoylcholine by the dart sac

(Activity expressed as µl. CO₂ evolved during 30 min. calculated for 100 µl. blood and 100 mg. tissue.)

		Solution (Table 2)					
		1	2	3	4	5	6
Blood:	Benzoylcholine	27	20	10	0	0	0
	Acetyl­aneurin	—	240	204	70	47	11
Dart sac:	Acetyl­methylcholine	82	74	39	29	16	11
	Benzoylcholine	33	27	16	14	10	—

Fig. 4. Comparison of the activity-pS curves of the systems *Helix* blood-acetylcholine and *Helix* blood-acetyl­methylcholine. The 30-min. values are calculated with the value of solution no. 3 as 100.

Fig. 5. Comparison of the activity-pS curves of the systems dog brain-acetylcholine and horse erythrocyte-acetylcholine (upper curves), dog brain-acetyl­methylcholine and horse erythrocyte-acetyl­methylcholine (lower curves). Full-drawn lines refer to brain, broken lines to erythrocytes.

also obtained this depressing effect with acetyl­methylcholine. The optimum concentration of the latter appears to be somewhat higher than that of acetylcholine, with respect to cholinesterase of brain and erythrocytes (Fig. 5).

The reaction curves are very similar in shape. For the rest, cholinesterase in brain and erythrocytes have properties very much alike (cf. Augustinsson, 1945). In both cases, the enzymes are more active

Fig. 6. Enzymic hydrolysis of acetylcholine by dart sac extracts containing 25.5 mg. tissue/0.4 ml. suspension. The numbers refer to the solutions in Table 2.

towards acetylcholine than towards acetylmethylcholine. This confirms the results of Nachmansohn & Rothenberg (1945), but it is at variance with that of Alles & Hawes (1940) who claim that 'the cell [red blood cell] enzyme is more active towards acetyl- β -methylcholine than towards acetylcholine'.

Cholinesterase activity of the dart sac. The pre-

liminary experiments have already shown that a striking difference exists between the enzymes of the snail blood and of the dart sac. To prove this further, the cholinesterase activity was measured at various substrate concentrations. The enzyme of the dart sac is not inhibited by excess of the substrate acetylcholine (Fig. 6), acetylmethylcholine or benzoylcholine (Table 4). On the other hand, the activity towards acetylmethylcholine is greater than towards benzoylcholine. Mendel & Rudney (1944), however, have claimed that the 'depressing activity with rising substrate concentrations should not be considered an inherent property of the specific enzyme. Specificity alone remains the true criterion for a differentiation of cholinesterases.'

Inhibition of cholinesterase of Helix blood and of the dart sac by physostigmine. It has been found on several previous occasions that the action of drugs or ions on cholinesterase varies according to the material used. Thus, quaternary-N bases inhibit the enzyme of red blood cells much more than that of the serum (man); the contrary is the case with tertiary-N bases (Sanz, 1945). Caffeine inhibits the specific cholinesterase and not the unspecific enzyme (Zeller & Bissegger, 1943; also Nachmansohn & Schneemann, 1945). Physostigmine inhibits the cholinesterase of *Helix* blood and of the dart sac (cf. Ammon, 1943). Surprisingly, this inhibition seems to be of the same magnitude as that of horse-serum enzyme. Table 5 gives the results of some

Table 5. Inhibition of the cholinesterase activity of *Helix* blood and of the dart sac by physostigmine salicylate, compared with that of horse serum

Tissue	Substrate	μ g. inhi- bitor	b_{50}		% inhi- bition
			No inhi- bitor	With inhi- bitor	
Blood	Acetylcholine*	0.3	205	33	84
	Acetylmethylcholine	0.3	89	15	83
Serum	Acetylcholine*	0.3	50	2	95
Dart sac	Acetylcholine*	0.2	273	128	53
Serum	Acetylcholine*	0.2	101	30	70

* Solution 3 (Table 2).

preliminary experiments carried out with physostigmine salicylate. The inhibitions of the hydrolysis of acetylcholine and acetylmethylcholine by the snail blood are equal. More experiments of this kind are being carried out.

DISCUSSION

The results of this investigation suggest that it is inadvisable to make the generalization that only two distinct acetylcholine-hydrolyzing enzymes exist. We now know of two types of activity with

respect to behaviour towards acetylcholine. Whether this is generally true for all forms of animal life is, however, far from being established.

There is nothing remarkable in the fact that an enzyme or enzymes hydrolyzing acetylcholine, can also split an ordinary ester, e.g. methyl butyrate. From the physiological point of view, however, it is important that the enzyme in the nerve tissues should be specific towards choline esters (Nachmansohn & Rothenberg, 1945). The biochemist, on the other hand, wishes to acquire more facts concerning the difference between the natures of the specific and the unspecific cholinesterase. This difference is more striking between the enzymes of the blood and the dart sac of *H. pomatia* than has previously been found in any other material. The specificity towards acetylcholine of the dart sac is very distinct, and it is quite different from that of any other specific cholinesterase from other sources, with respect, for example, to increase in the concentration of acetylcholine. The blood enzyme also has interesting properties. The different behaviours of the enzyme towards acetylcholine and acetylmethylcholine must be investigated more fully in order to find an explanation. The great affinity for acetylcholine is surprising.

We may conclude that the problem of the specificity of cholinesterase is not solved with the hypothesis of two types of cholinesterase. More detailed studies must be performed with purified enzyme preparations. The use of impure enzyme solutions is, of course, to the prejudice of the results presented above.

SUMMARY

1. An investigation of the cholinesterase activity of various tissues of *Helix pomatia* has been performed. A mixture of enzymes seems to be present in most cases.

2. The blood and the dart sac are especially active towards acetylcholine.

3. The blood enzyme splits acetylcholine at a high rate, as distinct from that of the dart sac. On the other hand, the sac enzyme is very active towards tributyrin. Neither enzyme splits ethyl acetate.

4. A striking difference is found between the enzymes of the snail blood and of the dart sac in their relative activities on acetyl- β -methylcholine.

5. With respect to the hydrolysis of acetylcholine as a function of substrate concentration, the enzymes of the blood and of the dart sac behave quite differently, excess of substrate inhibiting the activity of the blood enzyme, and not that of the sac enzyme. The hydrolysis of acetyl- β -methylcholine is in neither case depressed by high substrate concentration.

6. The results are compared with the values obtained for the hydrolysis of acetylcholine and acetyl- β -methylcholine by cholinesterases of brain (dog) and red blood cells (horse).

7. The inhibiting action of physostigmine on the cholinesterases of the snail blood and dart sac is shown in a preliminary experiment.

8. A more detailed determination of the autolysis of the substrates as a function of concentration is reported.

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