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SHORT COMMUNICATIONS

Some properties and the axonal transport of choline acetyltransferase in nervous tissue of the snail *Helix pomatia*

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Choline acetyltransferase (ChAc, EC 2.3.1.6), the enzyme which catalyses the formation of acetylcholine from choline and acetyl–CoA, has been studied in a variety of vertebrate tissues of both nervous [1, 2, 5] and non-nervous origin. Information on the properties of ChAc from invertebrate tissues is comparatively sparse, although the recent publications of Emson *et al.* [6] and Husain and Mautner [7] have contributed considerably to our knowledge and it would now appear (for discussion see Emson *et al.* [6] and McCaman and McCaman [1]) that ChAc in invertebrates differs in some respects from that in vertebrates. Moreover, studies on certain invertebrates nervous systems (viz. snail *Helix* and *Aplysia*) have shown clearly that ChAc is specifically localised in neurons containing the transmitter acetylcholine [9–12]. The present investigation is aimed at contributing further to our knowledge of ChAc in invertebrates or, more particularly, the snail *Helix pomatia*. It includes additional information on the characteristics of the enzyme as well as confirming earlier studies. Moreover, information is provided for the first time on an invertebrate system of ChAc transport, where nerves received a single or double ligature.

Various organs from freshly caught snails (*Helix pomatia*) were homogenised in ice-cold 10 mM EDTA, pH 7.4 (5% w/v) containing 0.5%. (v/v) Triton X-100 to ensure release of the enzyme ChAc. After centrifugation at 5000 *g* for 15 min, the supernatant was removed and used as the crude enzyme source.

The procedure used to analyse ChAc activity was as described by Fonnum [13] and the acetyl–CoA concentration employed to determine the K_m values was similar to that used by Emson *et al.* [12]. The incubation medium contained (final concentrations): 0.2 mM [1-^{14}C]acetyl–CoA (New England nuclear, sp. act. 54 mCi/m-mole), 300 mM NaCl, 50 mM sodium phosphate buffer (pH 7.4), 8 mM choline chloride, 20 mM EDTA (pH 7.4) and 0.1 mM physostigmine. The labelled acetyl–CoA was diluted with unlabelled compound obtained from Schwarz Mann (New York). A quantity of enzyme solution (2 μl) was placed in a small tube and substrate mixture added (5 μl). The solution was thoroughly mixed and incubated for 15 min at 37°. The enzymatic reaction was then terminated by placing the tube in a scintillation vial and washing it out with 5 ml sodium phosphate buffer. To the vial was then added 2 ml acetonitrile containing 10 mg Kalignost and 10 ml scintillation mixture (Scintimix in toluene, 5.5 g/l). The vial was shaken slightly and counted after allowing it to equilibriate in the scintillation counter for 15 min.

In order to study the transport of ChAc, the visceral nerve was ligatured as described previously [14]. Most of the shell of the animal was first removed and the snail pinned to a layer of wax at the base of a dissecting dish. An incision was then made along the dorsal surface of the animal so that the visceral nerve could be ligatured at one or two points (1 cm apart). In each case a thin glass rod was placed alongside the nerve and tied together securely with a fine silk thread. After the operation, the animals were completely covered with snail saline [15] which was continuously aerated and changed. Operated animals were kept for a maximum of 48 hr before removing the nerve to analyse portions of approximately 4 mm for ChAc activity.

Some general information on the nature of snail ChAc is shown in Table 1 and confirms the findings of others which

Table 1. Some characteristics of snail ChAc

	ChAc activity (μmole/mg wet weight tissue/60 min)	
Ganglia homogenate	0.0140 ± 0.004	($n = 15$)
17,000 *g* supernatant	0.0148 ± 0.003	($n = 15$)
17,000 *g* mitochondrial pellet	0.0039 ± 0.003	($n = 15$)
0–20% $[NH_4]_2SO_4$ fraction of 50,000 *g* supernatant	0.0162 ± 0.005	($n = 15$)
40–60% $[NH_4]_2SO_4$ fraction of 50,000 *g* supernatant	0.0092 ± 0.001	($n = 15$)
Homogenate from heart, hepatopancrease or albumin gland		(n at least 6)

Each value is the mean ± S.E.M. the figure in brackets indicate the number of separate experiment.

Factors important for maximal enzyme determination ($n = 5$): pH of 7, temperature of 40°, NaCl 300 mM or KCl 300 mM.

Enzyme determination, not maximal (n at least 4) when: pH <or> than 7, temperature <or> than 40°, NaCl <300 mM or KCl <300 mM and by less than 50 mM of either $MgCl_2$ or $CaCl_2$.

50 mM of following substances stimulate enzyme production by about 15 per cent ($n = 5$): histamine, imidazole and 5 hydroxytryptamine.

Table 2. Substrate specificity of snail ChAc

Substrate	Snail CNS		Torpedo electric organ*		Rat brain†		Squid head ganglia‡	
	K_m	RV	K_m	RV	K_m	RV	K_m	RV
Choline	0.37	1	11.5	1.0	0.17	1.0	0.66	1
Monoethylcholine	1.50	0.65	17.4	0.83	1.26	1.02	—	—
Diethylcholine	1.66	0.24	35.3	0.51	9.66	0.70	—	—
Triethylcholine	3.12	0.14	31.0	0.09	25.00	0.70	—	—
Pyrrolcholine	1.72	0.39	35.1	0.25	6.65	0.53	6.36	0.15

K_m values in mM. RV = relative value of V_{max}.
* Values from Baker and Dowdall [17].
† Values from Barker and Mittag [21].
‡ Values from Barker *et al.* [25].

show that the characteristics of the enzyme are not unlike those of the vertebrate enzyme [6, 9, 16]. Some distinctive features are, however, associated with the snail enzyme. For example, the unusual temperature co-efficient was previously discovered by others [6, 16] and is corroborated in this study.

Snail ChAc is also slightly but significantly stimulated by a number of amines and imidazoles but not by amino acids. This would suggest a possible interregulation between various transmitter amines and acetylcholine. The results are unlike those found for ChAc of the *Torpedo* electric organ [17] where both histamine and imidazole were found to inhibit enzyme activity. However, the enzyme from bovine caudate nucleus [18], behaves like the snail enzyme and is stimulated by histamine and imidazole.

Like the mammalian brain ChAc [13, 19, 20], snail ChAc is fairly specific for choline, judging from the K_m (apparent) value (Table 2). A similar K_m value for the snail enzyme was found by Emson *et al.* [6], i.e. 370 μm, and this differs considerably from the high K_m value (7.5 mM) found for *Aplysia* ganglia [9]. Moreover, like other enzyme sources, snail ChAc shows a clear discrimination in binding various analogues of choline (based on the K_m values) and a direct relationship between the square of the molecule radii and V_{max} values of the ethyl analogues of choline (Table 2 and Fig. 2). This relationship suggests a direct correspondence between the lowering of the binding energy of the positively charged substrate to the active site of the enzyme and the replacement of ethyl groups in the substrate [22].

The transport of ChAc in the ligated visceral nerve showed that the enzyme was transported very slowly. The main finding was that 48 hr after ligaturing the visceral nerve there was an increase of about 40 per cent in ChAc in the first 4 mm part immediately proximal to the ligature. After 16 hr the ChAc activity in this region above the ligature was not significantly greater than that of the controls, but after 24 hr there was an increase of about 20 per cent. In parts of the nerve distal to the ligature, no significant change in the enzyme activity was observed, although a downward trend was apparent. These experiments suggest that there is a proximal–distal transport of enzyme and this is strongly supported by the experimental results from nerves which received two ligatures (see Fig. 2). In nerves which received a double ligature, there was an increase in ChAc activity after 48 hr of about 40 per cent proximal to the proximal ligature, as was also reported for the single ligatured nerves. There was statistically no difference in enzyme activity in parts of nerve immediately distal to the proximal or distal ligatures (see Fig. 2). After 24 hr the ChAc activity proximal to the proxi-

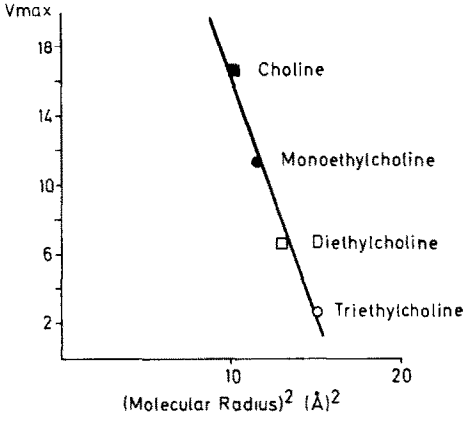

Fig. 1. Relationship between maximal rate (V_{max}) of acetylation of choline and its analogues and the square of the molecular radius. Values of V_{max} are taken from the curves used to determine the K_m's. The molecular ratios were determined as described by Hemsworth and Smith [22] and Baker and Dowdall [17]. Choline = 3.17 Å; monoethylcholine 3.35 Å; diethylcholine 3.57 Å; triethylcholine 3.8 Å.

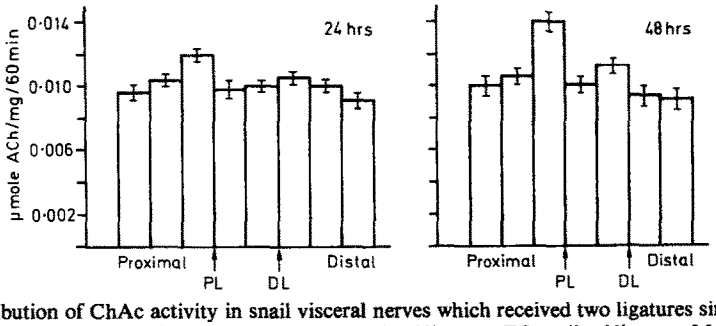

Fig. 2. Distribution of ChAc activity in snail visceral nerves which received two ligatures simultaneously. Arrows indicate the positions of the ligatures. PL = proximal ligature; DL = distal ligature. Means ± S.E.M. are shown where $n = 5$. The times indicate the interval between ligaturing the nerve and its removal from the snail.

mal ligature was also increased, but no alteration was observed on either side of the distal ligature (see Fig. 2). This supports, therefore, the hypothesis of a proximal–distal transport and that retrograde transport of the enzyme does not occur. However, any surmises cannot be conclusive, for it is important to note that enzyme activity is not diminished distal to a ligature, which would be consistent with a sole proximal–distal or orthograde transport process for ChAc. It may well be that ligaturing nerves for periods of longer than 48 hr would resolve this question. Although rates of orthograde transport for ChAc in the snail were not calculated, it is clear that it is a slow process, since transport could not be detected 24 hr after ligation, and after 48 hr the enzyme activity in the first proximal segment of the analysed nerve was only 40 per cent than the control. The transport of ChAc in snail nervous tissue is thus, in principle, similar to that described for rabbit sciatic nerve [23] and mammalian cholinergic nerves [24].

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Stereospecificity and active site requirements in a diisopropylphosphorofluoridate-hydrolyzing enzyme

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Squid nerve and hepatopancreas contain an enzyme that specifically hydrolyzes diisopropylphosphorofluoridate (DFP) [1]. This squid type DFPase is present in these tissues in other cephalopods—cuttlefish, nautilus, octopus—but is missing or present at markedly lower levels in other tissues and other classes of invertebrates [1–3]. A mammalian DFPase [4] appears to be a distinctly different enzyme, a major criterion being that the mammalian enzyme hydrolyzes another organophosphate, ethyl *N,N*-dimethylphosphoramidocyanidate (Tabun), much faster than DFP, whereas the order is reversed for squid type DFPase [1, 5]. Since DFP, Tabun, and their various analogues [6] are not naturally occurring compounds, the natural substrate for DFPase remains an important question. This is especially so for squid type DFPase because of its presence in nerve where it might have seemed, illogically, to be providing a protection against these powerfully neurotoxic agents [7].

Evidence has been presented showing Tabunase (as it was termed) in rat serum to be stereospecific, with the phosphorus atom the center of asymmetry [8]. This was confirmed [9], although the original observation and the confirmation were either indirect or at the limits of significance for the methods then available. We have now returned to this question with a different enzyme, namely squid type DFPase, a different substrate, and instrumentation for recording optical rotation in the millidegree range at wavelengths more favorable than the sodium D line of 589 nm. Some enzyme–inhibitor studies have also been made. The results suggest some structural requirements for the natural substrate, which would thus provide a physiological role for this otherwise seemingly functionless enzyme.

Squid type DFPase also hydrolyzes 1,2,2-trimethylpropyl methylphosphonofluoridate (Soman) [5], although with a K_m about ten times that for DFP [10], but does not hydrolyze