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THE PENETRATION OF ACETYLCHOLINE INTO THE CENTRAL NERVOUS SYSTEM OF THE COCKROACH *PERIPLANETA AMERICANA* L.

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INTRODUCTION

According to Treherne & Smith (1965*a*) acetylcholine penetrates rapidly into the tissues of the abdominal nerve cord of the cockroach *Periplaneta americana* L. This conflicts with the findings of Twarog & Roeder (1957), O'Brien (1957), and O'Brien & Fisher (1958), who concluded that there is a barrier to the passage of acetylcholine into the central nervous system of *P. americana*; Lord, Molloy & Potter (1963) also concluded there was a similar barrier in the housefly, *Musca domestica*. The disagreement may arise from the use of different techniques. Thus Treherne & Smith (1965*a*, *b*) measured directly the passage of ^{14}C-labelled acetylcholine into the cockroach central nervous system and concluded that there was no barrier to its entry, but Lord *et al.* (1963) measured the hydrolysis of acetylcholine by whole and homogenized nerve tissues from the housefly and using it as a measure of the penetration of acetylcholine into the tissues inferred that there was a barrier to the penetration of acetylcholine. The evidence for such a barrier was strengthened by the increased rate of hydrolysis following treatment with acetone.

Because a large difference in the permeability to acetylcholine of nerve tissue from the two species would have great significance in the study of nerve physiology and might affect the action of drugs on the nervous system, tests were made to resolve the apparent discrepancy between the results of different workers. The hydrolysis of acetylcholine by cockroach central nervous tissue was measured and used as an indication of the ability of the substrate to reach cholinesterase in the tissue after treatment with acetone or after mechanical destruction of the cellular structure, in conditions similar to those used for the housefly (Lord *et al.* 1963). The results of the biochemical tests were confirmed and amplified using histochemical methods, which employed acetylthiocholine, a cholinesterase substrate closely related to acetylcholine.

MATERIAL AND METHODS

The cockroaches used were adult males taken 3–7 weeks after their final moult. Parts of the ventral nerve cord used for biochemical tests were dissected from live decapitated cockroaches under the saline of Yamasaki & Narahashi (1959). They were placed, either whole or after removal of the nerve sheath, in ice-cold saline to await cholinesterase estimation, either without further treatment or after standing for 2 hr. in

acetone at −20° C. The nerve sheath was removed by a method similar to that of Twarog & Roeder (1956). The sheath in the middle of the dorsal or ventral surface of a ganglion was grasped with two pairs of finely ground watchmakers' forceps and gently torn apart. It was then peeled from the ganglion and connectives, usually coming away in one piece. Paraffin sections of desheathed ganglia, fixed in alcoholic Bouin and stained in Ehrlich's haematoxylin and eosin, showed that this method, unlike that of Twarog and Roeder, which removed both the connective tissue sheath (neural lamella) and the underlying perineurium cells, removed mainly the neural lamella, leaving the perineurium cells over much of the ganglia intact.

Cholinesterase activity was measured in Warburg manometers at 25° C. in an atmosphere of 5% CO_2 and 95% N_2, using 3 or 10 mM acetylcholine chloride as substrate. Micro-flasks with a total volume of approximately 7 ml. contained a total reaction volume of 1·5 ml., including 0·02 M-$NaHCO_3$ and 0·2 M-NaCl. Homogenates were prepared by grinding the tissues with 1 ml. of ice-cold 0·4 M-NaCl in a power-driven, all-glass homogenizer. A 0·75 ml. portion was assayed.

For histochemical tests a method was used similar to that described by Burt, Gregory & Molloy (1966). Ganglia were dissected from live, chilled cockroaches under 0·85% sodium chloride solution and were tested for cholinesterase activity by the Gomori (1952) thiocholine method, either with or without pre-treatment for at least 2 hr. with acetone at −20° C. Ganglia, whole or desheathed, or their sheaths, were incubated in Gomori buffer containing 0·005 g. of substrate (acetylthiocholine iodide) per 5 ml. for 45 min. at 25 ± 1° C. and pH 6·1. To check the depth of penetration of staining, some ganglia (after incubation, dehydration and clearing) were embedded in Paramat (G. T. Gurr) and sectioned at 10 μ. They were mounted without further staining. The distribution of staining was made more readily visible by increasing the incubation time to 225 min., which increased the intensity of staining without apparently affecting its depth of penetration.

RESULTS

Biochemical. The rate at which acetylcholine was hydrolysed by cholinesterase in the nerve tissue was used to indicate the ability of the substrate to reach the enzyme. To determine the effects on the rate of acetylcholine hydrolysis of treating parts of the cockroach ventral nerve cord with acetone at −20° C. or destroying the cell structure by grinding, two series of tests were made. In one series ganglia II and III from the thoracic nerve cord were used, in the other abdominal ganglia I to VI. In both series a part of only one nerve cord was tested in each Warburg manometer. The results are summarized in Table I. The intact nerve cord, untreated with acetone, hydrolyses acetylcholine only slowly, but destroying the cells by grinding increases the rate of hydrolysis about 20-fold. Treating intact nerve cords with acetone increases their rate of acetylcholine hydrolysis five to ten times, and grinding the cords after treatment with acetone about doubles the rate. However, the rate of hydrolysis by ground, acetone-treated cords is less than that of ground cords not treated with acetone; this suggests that the acetone treatment partly inactivates the cholinesterase. Removing the sheath from untreated thoracic nerve cord increases the rate of hydrolysis two to sixfold, almost as much as treating the cord with acetone, but histochemical evidence

Table 1. *The effect of various treatments on the hydrolysis of acetylcholine by parts of the ventral nerve cord of* Periplaneta americana

Treatment	Hydrolysis of acetylcholine (mμM/cord/10 min.)	
	3 mM acetylcholine	10 mM acetylcholine
Thoracic nerve cord (ganglia II and III)		
Untreated		
Whole	70, 62, 41	9, 20, 66
Ground	1010, 986, 894, 865	
Desheathed	160	165, 240
Acetone-treated		
Whole	236, 232	—
Ground	560, 761	—
Desheathed	143†, 124†, 237*	—
Abdominal nerve cord (ganglia I–VI)		
Untreated		
Whole	49, 57, 21, 38	16, 14, 42
Ground	834, 768	—
Acetone-treated		
Whole	282, 290, 349, 286, 245, 214	—
Ground	507, 548	—

† Treated with acetone before removing sheath.
* Treated with acetone after removing sheath.

indicates that this increase probably results from damage to small nerves and the superficial cells of the connectives during desheathing. Treating the cord with acetone before or after desheathing does not seem to change the rate of hydrolysis of acetylcholine greatly. Sheaths removed from the thoracic nerve cord hydrolysed acetylcholine slowly, at about the same rate as whole untreated thoracic cord, but this activity was probably due to small pieces of damaged nerves attached to them.

The last abdominal ganglion was examined to obtain results directly comparable with those of Treherne & Smith (1965*b*). It seems to hydrolyse acetylcholine about as quickly as the rest of the abdominal cord (Table 2), and the sum of the two rates equals the rate for the whole abdominal cord (Table 1). Removing the sheath from the last abdominal ganglion about doubles the rate of hydrolysis (Table 2). Hydrolysis of acetylcholine by the sheath from the last abdominal ganglion could not be detected.

Histochemical. Histochemical tests showed that whole ganglia untreated with acetone stained only at the cut ends of nerves and at other sites where cells had been mechanically damaged. The ganglia themselves were unstained because their superficial layers (perineurium cells, glial cells and neuron cell bodies), except for certain of the neuron cell bodies, contain relatively little cholinesterase and the acetylthiocholine evidently did not penetrate to the cholinesterase abundant in the central neuropile. In the nerves damaged cells were intensely stained even though the neural lamella appeared intact, so that here at least acetylthiocholine seems able to penetrate the neural lamella. It does not, however, seem able to penetrate undamaged cells, untreated with acetone, sufficiently rapidly to show histochemically.

Removing the sheath had little effect on the histochemical staining of the ganglia, for although it damaged the superficial cells these contain too little cholinesterase to

Table 2. *Hydrolysis of acetylcholine by parts of the abdominal nerve cord of* Periplaneta americana

Treatment	Hydrolysis of 10 mM acetylcholine (mμM/cord/10 min.)
Abdominal ganglia I–V	
Whole	29, 14
Last abdominal ganglion (VI)	
Whole	20
Desheathed	66, 37

show histochemically in whole mounts. There was no staining in localized areas to suggest increased penetration of acetylthiocholine in places where the perineurium had been removed with the neural lamella. Removing the sheath of the connectives increased the superficial staining, for here the damaged cells do contain demonstrable amounts of cholinesterase. The isolated sheath did not stain at all, except where small pieces of damaged nerves adhered to it.

Whole ganglia treated with acetone stained well, but paraffin sections showed that they were not stained throughout. The nerves and connectives were stained only in the outer 6–9 μ, beneath the unstained neural lamella. Staining in the ganglia was restricted to a zone, 30–60 μ wide, around the periphery. It was concentrated mainly at the edge of the neuropile but there was some staining in the glial cell layer, especially of the cholinesterase-rich neuron cell bodies.

DISCUSSION

The biochemical tests show that treating the intact cockroach nerve cord with cold acetone increases the rate of hydrolysis of acetylcholine by the cholinesterase in the tissue, which suggests that acetone destroys a barrier to the free penetration of acetylcholine into the tissue. The increased rate of hydrolysis observed when tissue structure is destroyed by grinding is further evidence to support this suggestion. The alternative explanation, that acetone activates the cholinesterase, is contradicted by the fact that ground, acetone-treated cords hydrolyse acetylcholine more slowly than ground, untreated cords, which suggests that acetone inactivates some of the cholinesterase.

The histochemical results support these conclusions, for though untreated cords showed little cholinesterase activity, after treatment with acetone much activity could be demonstrated. Even after acetone treatment only the outermost layers of the tissue were stained, although work to be published elsewhere has shown that cholinesterase is abundant throughout most of the nerve tissue. The limited penetration of staining is probably due to the substrate entering slowly enough for the cholinesterase in the tissue to decompose it before it can penetrate deeply.

The histochemical evidence indicates that the barrier to the penetration of acetylcholine into untreated nerve cords lies in the cells themselves rather than in the connective tissue sheath of the cord, because desheathing has little effect on the penetration of substrate. The main factor facilitating entry of substrate into untreated cords seems to be cell damage, because the histochemical tests demonstrated cholinesterase activity only in damaged cells, and did so even when the neural lamella appeared

intact. This would explain the biochemical results, for grinding the nerve cords, which obviously must destroy the cell structure, greatly increased the rate of acetylcholine hydrolysis.

Because acetone treatment increases the rate of hydrolysis of acetylcholine (Table 1), it must facilitate the penetration of acetylcholine into the tissue. Equally, some limitation to the penetration of acetylcholine must still remain after acetone treatment, because grinding acetone-treated tissues further increases the hydrolysis of acetylcholine (Table 1). This is in contrast to housefly central nervous tissue, which hydrolyses acetylcholine as quickly after acetone treatment as when ground, and whose cholinesterase, unlike that of the cockroach, is not inactivated by acetone. The apparent permeability of nerve tissue to acetylcholine may be increased by acetone less in the cockroach than in the housefly, either because the central nervous system of the cockroach is bigger, or because it is less permeable after acetone treatment. Difference in size is the more likely explanation, for substrate diffusing into cockroach ganglia must travel farther than in housefly ganglia, which could result in an apparently lower activity. Difference in size could also account for the partial inactivation of cholinesterase in cockroach but not housefly nerve tissue, for cooling and dehydration by acetone, both processes likely to damage cholinesterase, must take longer in larger organs.

Desheathed cockroach nerve cords and acetone-treated cords hydrolyse acetylcholine at about the same rate. This seems to be fortuitous and without physiological significance because, as already noted, the hydrolysis of acetylcholine in desheathed cords results from damage to small nerves and superficial cells of connectives during desheathing, but in acetone-treated cords it is determined in part by increased permeability to acetylcholine and in part by the extent to which cholinesterase is inactivated.

The entry of acetylcholine into cockroach nerve cords therefore seems to be restricted, a conclusion differing from that of Treherne & Smith (1965*a*), who stated that acetylcholine penetrates rapidly into cockroach nervous tissue. However, close inspection of their data and those presented here suggests that the disagreement is mainly in terminology or interpretation. After measuring directly the penetration of acetylcholine into cockroach nerve cords, Treherne and Smith considered the cords permeable to acetylcholine, whereas we find that penetration is considerably increased by various treatments and therefore deduce a barrier to it.

From Table 1 the minimum and maximum rates of hydrolysis of acetylcholine by whole cockroach nerve cord can be calculated as 1·3 and 12 mμM acetylcholine per min. per cord, or approximately 0·2 to 2·0 mM acetylcholine per kg. cord tissue per min. (The cords used in our experiments weighed about 7 mg.) Strictly comparable figures are not available from Treherne and Smith's papers, but by using the values at 5 min. and 1 min. from their graph (text-fig. 5, Treherne & Smith, 1965*b*), a minimum and maximum rate of entry of acetylcholine into eserinized tissues can be estimated as between 0·6 and 2·0 mM acetylcholine per kg. tissue per min. Thus if the rate of hydrolysis of acetylcholine is taken to be a measure of the penetration of acetylcholine into the insect ganglia and the outward diffusion of acid products, the rate of penetration calculated from Table 1 agrees closely with the values obtained by the more direct methods of Treherne and Smith. (The close agreement is remarkable

because the values for the rates of hydrolysis of acetylcholine are very close to the limits of assay possible with Warburg manometers and therefore can be only very approximate.)

The present position may therefore be summarized as follows. Whether or not the normal rate of penetration of acetylcholine into the insect nervous system is considered rapid, it is clearly restricted in some way, for the rate can be much increased by treating with acetone. Penetration of untreated ganglia by acetylcholine is probably limited to the extracellular diffusion pathways in the perineurium and underlying tissues (Smith & Treherne, 1963; Maddrell & Treherne, 1966). Because acetylthiocholine fails to penetrate into desheathed ganglia, even where the perineurium has been removed, but penetrates to the edge of the neuropile in acetone-treated ganglia, the restriction seems to be situated in the glial cell layer. Acetylthiocholine probably fails to penetrate far into the neuropile in acetone-treated ganglia because it is rapidly decomposed by the very active cholinesterase in that region. These conclusions are supported by those of Wigglesworth (1958), who found that acetylthiocholine hardly penetrated deeper than the perineurium of whole ganglia of *Rhodnius prolixus*, even when they were fixed in formaldehyde, and that when ganglia were cut across the staining reaction was confined to the cut surface, but showed most cholinesterase activity in the neuropile. Therefore synapses in the ventral nerve cord of the cockroach are probably protected from extraneous acetylcholine not only by a high level of cholinesterase activity (Treherne & Smith, 1965*b*), and possibly by very local barriers (Treherne & Smith, 1965*a*), but also by a barrier outside the neuropile probably situated in the glial cell layer.

SUMMARY

1. The hydrolysis of acetylcholine and acetylthiocholine by cockroach ventral nerve cords was examined by biochemical and histochemical methods, and used as an indication of the penetration of the substrates into the nerve cord.

2. The rate of penetration of acetylcholine into intact nerve cords, measured biochemically, agrees well with estimates by Treherne & Smith (1965*b*) using a more direct method.

3. A barrier to the penetration of the two cholinesterase substrates is destroyed by treatment with acetone, which increased the rate of hydrolysis of acetylcholine five- to tenfold.

4. Histochemical tests showed that acetylthiocholine is probably prevented from penetrating freely to the neuropile of the ganglia by the glial cell layer. When the restriction is abolished by treating the cord with acetone, acetylthiocholine reaches the neuropile but penetrates it for only a short distance, probably because it is decomposed by the cholinesterase in this region.

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