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# Micro-colorimetric determination of cholinesterase activity of motor end plates in the rat diaphragm

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A spectrophotometric method is described for the determination of total cholinesterase activity in groups of 3–30 end plates, after staining with copper thiocholine and dissecting from the rat diaphragm, by continuous recording of the yellow colour (412 mμ) produced by reaction between dithiobisnitrobenzoate (1mM) and thiol groups liberated enzymatically from acetylthiocholine (5mM) at pH 7·0. Cholinesterase activity of end plates after correction for muscle ranged from $3{\cdot}3–22{\cdot}3 \times 10^{-11}$M/end plate/hr and muscle cholinesterase activity ranged from $8{\cdot}8–29{\cdot}4 \times 10^{-11}$ μg/hr. 2·9–26·7% of measured end plate cholinesterase activity was attributable to muscle. The results for end plate cholinesterase agreed with those obtained by other workers using microgasometric techniques. It was calculated that there were approximately $3{\cdot}6 \times 10^6$ cholinesterase active sites/end plate which compared closely with $6 \times 10^6$ molecules acetylcholine released/nerve ending/impulse and $2{\cdot}6 \times 10^6$ cholinergic receptors/end plate given in the literature. It is suggested that each acetylcholine molecule after liberation from a nerve ending may interact with one receptor and be destroyed by one cholinesterase active site.

TOTAL cholinesterase activity, that is acetylcholinesterase + butyrylcholinesterase, was determined in groups of end plates dissected from the rat diaphragm which had been stained by a modification of the histochemical procedure for cholinesterase (Koelle & Friedenwald, 1949). Acetylthiocholine was used as a substrate and the thiol groups liberated from this were reacted with copper glycinate to give deposits of copper thiocholine (Malmgren & Sylvén, 1955) on the end plates. This provided a stain adequate for dissection purposes without further development with ammonium sulphide. The groups of end plates taken for cholinesterase determinations contained appreciable amounts of inseparable muscle tissue and therefore the cholinesterase activity of rat diaphragm muscle was also determined.

## Experimental

### REAGENTS

*Staining solution.* 5mM acetylthiocholine iodide (Sigma) prepared immediately before use in a mixture containing 5 ml 0·1M glycine, 5 ml 0·02M $CuSO_4$, 5 ml 0·5M $MgCl_2$, and 35 ml pH 6·8 maleate buffer (Gomori, 1955).

*Buffer.* pH 7·0 phosphate buffer prepared by mixing 28 ml of 0·2M $NaH_2PO_4$ and 72 ml of 0·2M $Na_2HPO_4$.

*Incubation mixture.* 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)/5 mM acetylthiocholine prepared by weighing 0·079 g DTNB (Kodak) and 0·0289 g acetylthiocholine iodide in the same beaker and dissolving immediately before use in 20 ml buffer.

### METHOD

Male Wistar rats weighing 120–170 g were decapitated and the diaphragms removed by cutting around the central tendon thus avoiding

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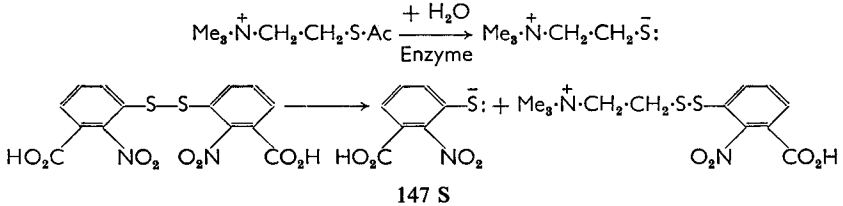
damage to major blood vessels. The diaphragm was then dissected from the ribs and placed in the staining solution for 10 min. The end plates appeared as dots in the characteristic horseshoe arrangement around the centre of the diaphragm. After staining, groups of 3–28 end plates were dissected from the left dorsal tip of the diaphragm under a dissecting microscope at 16× magnification using a scalpel (Swann-Morton, size 15 blade); the diaphragm was held firm by a needle attached to a Singer micromanipulator. Samples of muscle ranging in weight from 2·4–25 $\mu$g were similarly dissected from an area immediately adjacent to the end plates and subjected to the procedure as described for end plates.

After dissection the preparation was placed in a Petri dish and the end plates were counted accurately using 16× and 40× magnifications. By means of a glass rod with a tip approximately 0·2 mm in diameter the preparation was then transferred to a cavity slide containing incubation mixture: this rod was used for all manipulations of the preparations. When the preparation became a uniform yellow colour (5–10 min), it was assumed to be saturated with acetylthiocholine and DTNB and for the assay it was transferred to a microcuvette containing 100 $\mu$l or 200 $\mu$l incubation mixture. The preparation was placed on the meniscus and allowed to come to rest at the bottom of the meniscus before being gently thrust into the solution. This ensured that the preparation was always in approximately the same position at the bottom of the microcuvette and improved the reproducibility of the results.

Two groups of end plates, one muscle sample, and a reagent blank were used in each experiment. The four microcuvettes were placed in a Hilger-Gilford Reaction Kinetics Recording Spectrophotometer with the cell housing maintained at 24–25° and a recording was made over at least 30 min of the change in absorbance at 412 m$\mu$. It was found important to ensure that the light beam in the spectrophotometer passed through the whole height of liquid in each microcuvette. Repeat cholinesterase determinations were made by transferring the preparations to fresh incubation mixture. After estimation each preparation was washed with distilled water on a cavity slide and the water removed by suction. The preparations were then covered with a slide, dried overnight, and subsequently weighed on a quartz fibre fish pole balance (Lowry, 1953).

## CALCULATION OF RESULTS

The assay is based on the following reactions (Ellman, Courtney, Andres & Featherstone, 1961):



The enzymatic production of thiol groups from acetylthiocholine was approximately linear during the period of the experiments. Cholinesterase activities were therefore calculated from the slopes of the colour/time curves drawn on the spectrophotometer recordings. The slopes for the reagent blanks were subtracted to correct for non-enzymic hydrolysis. Three determinations were made for each preparation and the mean corrected slope was obtained from the individual corrected slopes: these did not differ by more than 6%. The amount of substrate hydrolysed was calculated as described by Ellman (1959) from the mean corrected slope and the molecular extinction coefficient ($\epsilon$13,600) for the yellow anion produced by the reaction between thiol groups and DTNB. That this coefficient was applicable under the conditions used was checked in control experiments with glutathione. Muscle cholinesterase activity was calculated as moles substrate hydrolysed/$\mu$g dry tissue/hr (M/$\mu$g/hr) and end plate cholinesterase activity, corrected for muscle, was expressed as moles substrate hydrolysed/end plate/hr (M/ep/hr).

## RESULTS

Cholinesterase activities of end plates and muscle were determined in eleven experiments on the diaphragms of different rats and the results are shown in Table 1. In experiments 9, 10 and 11 the reaction volume

TABLE 1. CHOLINESTERASE (CHE) ACTIVITIES OF END PLATES AND MUSCLE IN THE RAT DIAPHRAGM

| Expt. | No. of end plates | Weight ($\mu$g) | Measured end plate ChE activity (M $\times$ $10^{-11}$/hr) | Muscle correction (M $\times$ $10^{-11}$/hr) | Muscle ChE activity (M $\times$ $10^{-11}$/$\mu$g/hr) | Corrected end plate ChE activity (M $\times$ $10^{-11}$/ep/hr) |
|---|---|---|---|---|---|---|
| 1 | 9 | 0·4 | 66·2 | 6·5 | 16·2 | 5·6 |
| | 10 | 0·6 | 42·6 | 9·7 | | 3·3 |
| 2 | 12 | 0·37 | 133·8 | 7·6 | 20·6 | 10·5 |
| | 10 | 0·95 | 242·6 | 19·6 | | 22·3 |
| 3 | 8 | 0·44 | 138·2 | 8·4 | 19·1 | 16·2 |
| | 6 | 0·36 | 58·8 | 6·9 | | 8·6 |
| 4 | 11 | 0·98 | 95·6 | 8·6 | 8·8 | 7·9 |
| | 13 | 0·98 | 191·0 | 8·6 | | 14·0 |
| 5 | 11 | 0·63 | 148·5 | 18·5 | 29·4 | 11·8 |
| | 8 | 0·67 | 95·6 | 19·7 | | 9·5 |
| 6 | 15 | 0·48 | 111·7 | 6·3 | 13·2 | 7·0 |
| | 14 | 0·58 | 130·8 | 7·7 | | 8·8 |
| 7 | 28 | 4·18 | 326·3 | 43·1 | 10·3 | 10·1 |
| | 20 | 5·95 | 229·3 | 61·3 | | 8·4 |
| 8 | 12 | 1·1 | 164·6 | 21·0 | 19·1 | 11·8 |
| | 10 | 1·4 | 111·7 | 26·7 | | 8·5 |
| 9 | 5 | 0·19 | 25·7 | 2·2 | 11·8 | 4·7 |
| | 8 | 0·19 | 76·5 | 2·2 | | 9·3 |
| 10 | 4 | 0·4 | 72·8 | 4·4 | 11·0 | 17·1 |
| | 5 | 0·28 | 88·2 | 3·1 | | 17·0 |
| 11 | 5 | 0·21 | 62·5 | 5·9 | 27·9 | 11·3 |
| | 5 | 0·30 | 63·2 | 8·4 | | 10·9 |
| | 3 | 0·16 | 38·9 | 4·5 | | 11·5 |
| | | | | Mean | 17·0 | 10·7 |
| | | | | Standard Deviation | ± 6·99 | ± 4·3 |

was reduced from 200 to 100 $\mu$l which allowed the use of a smaller number of end plates. In experiment 11 three determinations were made alternately on three groups of end plates and three samples of muscle.

The Table shows that after correction for muscle the cholinesterase activity of end plates ranged from 3·3–22·3 $\times$ $10^{-11}$M/ep/hr and the cholinesterase activity of muscle itself ranged from 8·8–29·4 $\times$ $10^{-11}$M/$\mu$g/hr. When activities were expressed on a weight basis the proportion of the measured end plate cholinesterase activity attributable to muscle ranged from 2·9 to 26·7% (mean 11%). There was no relationship between the corrected end plate cholinesterase activity (M/ep/hr) and muscle cholinesterase activity (M/$\mu$g/hr).

## Discussion

The results obtained for end plate cholinesterase activity using the described method agree closely with the published data from microgasometric work when the latter were converted from $\mu$l $CO_2$ produced to moles substrate hydrolysed/end plate/hr. Thus the range of 9·9–28·6 $\times$ $10^{-11}$M/ep/hr (Giacobini & Holmstedt, 1960) for the total cholinesterase activity of end plates from the rat rectus abdominis, and the corresponding values of 14·3–20·1 $\times$ $10^{-11}$M/ep/hr and 22·3–53·6 $\times$ $10^{-11}$M/ep/hr for the mouse diaphragm and gastrocnemius respectively (Brzin & Zajicek, 1958) compare well with the range of results reported in this paper for the rat diaphragm (3·3–22·3 $\times$ $10^{-11}$M/ep/hr). It is interesting to note that Giacobini & Holmstedt (1960) gave results for unstained end plates since these could be visualised relatively easily in the rat rectus abdominis. On the other hand the results given by Brzin & Zajicek (1958) were obtained with end plates which had been stained by a similar method to that described for the rat diaphragm. It would therefore appear that the staining solution does not markedly affect the activity of the enzyme.

It is not known to what extent muscle cholinesterase contributed to the above results for endplate cholinesterase activity obtained by Giacobini & Holmstedt (1960) and Brzin & Zajicek (1958) although the results of our experiments indicate that muscle may contribute up to approximately 27% of the measured end plate activity. Corrected cholinesterase activities were expressed per end plate rather than on a weight basis since the weights of groups of end plates assayed were assumed to be due mainly to muscle. The sizes of end plates varied from 13·0–35·1 $\mu$ (length) and 6·5–22·1 $\mu$ (breadth) but no relation was observed between the dimensions and the cholinesterase activities of the end plates. However the complexity of end plate morphology may not permit calculation of the enzyme concentration per unit of surface area or volume since the anatomical structure resembles a tree more than a plate (Giacobini & Holmstedt, 1960).

The cholinesterase activity of muscle has been studied previously in some detail using short segments of single muscle fibres by means of a sensitive microgasometric technique (Giacobini & Holmstedt, 1960). Results which were given per unit volume of muscle cannot be compared

accurately with the results given in this paper for relatively large samples of tissue which may contain varying amounts of connective tissue and blood. A rough comparison is possible however between the cholinesterase activities of muscle ($8{\cdot}8$–$29{\cdot}4 \times 10^{-11}$M/$\mu$g/hr) and myosin (18 $\mu$g acetylcholine/mg protein/hr; $10 \times 10^{-11}$M/$\mu$g protein/hr) (Varga, König, Kiss, Kovaćs & Hegedüs, 1955) and it is therefore possible that myosin may contribute appreciably to the cholinesterase activity of muscle.

The mean result obtained for end plate cholinesterase activity is $10{\cdot}7 \times 10^{-11}$M/ep/hr and the number of cholinesterase active sites per end plate can be calculated approximately by dividing the end plate cholinesterase activity expressed as $10{\cdot}7 \times 10^{11}$ acetylcholine molecules/ep/min by the turnover number of $2{\cdot}95 \times 10^{5}$ acetylcholine molecules hydrolysed/min/acetylcholinesterase active site (Cohen & Warringa, 1953). The value obtained, $3{\cdot}6 \times 10^{6}$ cholinesterase active sites per end plate, compares closely with $6 \times 10^{6}$ acetylcholine molecules liberated/nerve ending/impulse obtained from the results given by Krnjević & Mitchell (1961), and also with the number of cholinergic receptors ($2{\cdot}6 \times 10^{6}$)/end plate determined by Waser (1962). Although these results have been obtained under widely different conditions they suggest that there are approximately the same number of receptors and cholinesterase active sites as the number of acetylcholine molecules released per nerve ending per impulse. If correct it may be assumed that after liberation from a nerve ending each molecule of acetylcholine interacts with one receptor and is destroyed by one cholinesterase active site.

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