ON THE HISTOCHEMISTRY OF THE MYONEURAL JUNCTION.

F. A. DENZ.

From the Medical Research Council Unit for Research in Toxicology, Woodmansterne Road, Carshalton Beeches, Surrey.

Received for publication February 16, 1953.

THE demonstration by a histochemical technique that an enzyme is active at a particular site is interesting in the promise it holds of ascribing function to microscopic structures. But this has been largely unfulfilled because the enzymes so localized have not been identified with the precision necessary to determine their function. Enzyme identification is the central problem in histochemistry and this paper describes the difficulties that are met when this problem is attacked. When the myoneural junctions of the rat diaphragm are shown up by the methods of Koelle (1950), and of Gomori (1948) for cholinesterase and of Nachlas and Seligman (1949*a*) for esterase it is apparent that the same structure is displayed by three methods using very different substrates. The problem is the definition in biochemical terms of the enzyme or enzymes responsible for these three pictures.

The fundamental limitation of histochemical methods lies in the choice of a substrate which must give on hydrolysis an insoluble product that can be converted into a coloured compound. A substrate that gives a good histological picture is usually so unnatural that the fact of its hydrolysis does not identify the enzyme responsible. Further, the known presence of an enzyme in a tissue does not necessarily mean that histochemical staining is due to this enzyme. The best histochemical staining is obtained where the enzyme is sharply localised and of high focal activity, and its overall activity in the tissue may be much less than that of a second enzyme distributed more generally. The difficulties raised by the presence of several enzymes similar in activity in the one tissue are notorious and this is the situation in the rat diaphragm. The enzymes are specific (or " true " or acetyl-) cholinesterase, non-specific (or " pseudo ") cholinesterase and ali-esterases (as defined by Richter and Croft, 1942) of which two, A- and B-esterase have been characterized by Aldridge (1953*a*). The problem can be illustrated by reference to the method of Nachlas and Seligman (1949*a*) for esterases in which β-naphthyl acetate is the substrate. Seligman and his colleagues have concluded that specific and non-specific cholinesterases as well as ali-esterases hydrolyse β-naphthyl acetate (Ravin, Tsou and Seligman, 1951; Barnnett and Seligman, 1951) and the staining may be due to any of these enzymes.

The method of Gomori (1948) is based on the hydrolysis of myristoyl choline. From the work of Adams and Whittaker (1948, 1949) it seems likely that myristoyl choline (containing a C_{14} acid radical) would be hydrolysed by non-specific rather

than by specific cholinesterase, and indeed Gomori found that two highly active preparations of specific cholinesterase did not hydrolyse myristoyl choline at all. These observations suggest nothing more than that the staining is due to an enzyme other than specific cholinesterase. The substrate for the method of Koelle (1950) is acetyl thiocholine and it is hydrolysed by both specific and non-specific cholinesterase. Koelle met this difficulty firstly by using DFP at a molar concentration of 10^{-6} to inhibit non-specific cholinesterase leaving the specific cholinesterase relatively unaffected, and secondly by using butyryl thiocholine as a selective substrate for non-specific cholinesterase. With these methods Koelle concluded that the skeletal muscle of the cat contains specific cholinesterase "almost exclusively." This conclusion is in conflict with the implications from the Gomori method and shows a need for an investigation of the specificity of the various methods.

In this paper these problems have been investigated in two ways.

1. The effect of selective inhibitors of specific and non-specific cholinesterase on histochemical staining has been studied. With each of the histochemical methods the concentration of inhibitor necessary to reduce staining to about half of normal intensity was found for the series of inhibitors and this pattern was compared with the known inhibitory powers of these compounds, against specific and non-specific cholinesterase, and as far as these are known, of aliesterase.

2. An attempt has been made to confirm the histological findings by biochemical means. The ability of active preparations of specific cholinesterase to hydrolyze myristoyl choline and β-naphthyl acetate has been studied. As the enzyme preparations are necessarily crude their effects may be due not to specific cholinesterase, but to other esterases present as contaminants. This point has been investigated by examining the effect of selective inhibitors on the activity of the enzyme preparations.

The present work was done as part of a study of the way in which organophosphorus compounds produce neuro-muscular block by inhibiting cholinesterase. The presence in muscle of several esterases capable of hydrolysing acetylcholine greatly complicates the study of events at the myoneural junctions in poisoning by anticholinesterases. This paper is concerned with the identification of these esterases and with their precise localization in the muscle of the rat diaphragm.

MATERIALS AND METHODS.

Rat diaphragm preparations.

The rats were killed by coal gas and the diaphragm removed in one piece by cutting close to the costal margin with scissors. The phrenic nerves sweep around the diaphragm from ventral to dorsal aspect and the myoneural junctions lie closely on either side of the nerves as they run at right angles to the long axis of the muscle fibres. If the excised diaphragm is held up to the light the course of the phrenic nerves can easily be seen. Pieces of diaphragm 6–10 mm. × 5 mm., were cut with scissors so that the phrenic nerve passed down the middle of the tissue which was then laid flat on the freezing head of the microtome. Frozen sections of the unfixed diaphragm were cut at 10–15μ. Each section contained a line of myoneural junctions, running down the middle of the section. The sections were floated out in water and were sufficiently robust to be manipulated with glass rods and passed through a series of solutions.

Many of the histological methods were modified so that frozen sections of fresh unfixed diaphragm could be used in place of the usual paraffin sections.

Gomori (1948) *method for cholinesterase.*

The prescribed buffer tris- (hydroxymethyl)-aminomethane was not obtainable in this country. A bicarbonate buffer was used instead. Fresh frozen sections were incubated for 30 min. in a mixture of 3 ml. 0·09 M sodium bicarbonate, 1 ml. 0·1 M cobalt acetate, 5 ml. distilled water and 1 ml. 0·02 M myristoyl choline. The mixture was gassed with 95 per cent N_2, 5 per cent CO_2 for 5 min. before use. After incubation the sections were washed in distilled water for 5 min., treated with dilute yellow ammonium sulphide, washed again, floated on to microscope slides, pressed flat with filter paper, passed through alcohol and xylol and mounted in Canada balsam. The substrate, myristoyl choline, was synthesised according to the method described by Gomori (1948).

Nachlas and Seligman (1949*a*) *method for esterase.*

This was modified by using Fast blue B (I.C.I.) instead of α-naphthyl diazonium naphthalene 1, 5-disulphonate as a coupling agent. Freshly cut frozen sections were placed in the substrate mixture at room temperature for 12 min., washed in distilled water and mounted on slides in glycerine jelly.

In the parallel experiments Gomori's (1950) modification was used in which α-naphthyl acetate is the substrate and the acetone is reduced to 0·5 ml. for each 10 mg. of substrate. Sections were placed in a freshly prepared and filtered solution of the substrate for 4 min. at room temperature. The sections were then washed in distilled water and mounted on slides in Farrant's medium.

Some diaphragms were fixed in cold acetone and embedded in paraffin according to the method of Nachlas and Seligman (1949*a*) and the sections were treated with the substrate as described in the original method.

Gomori (1945) *method for lipase.*

This method involving acetone fixation and paraffin embedding was followed for the diaphragm. The substrates were Tween 40 and 60. These are long chain fatty acid esters of substituted hexitans and hexides. In addition frozen sections of fresh diaphragm were incubated overnight in the substrate at 37°. They were washed in distilled water and mounted in glycerine jelly.

Koelle method for cholinesterase.

This method (Koelle and Friedenwald, 1949, Koelle, 1950) calls for fresh unfixed tissues. No improvement in intensity of staining or sharpness of localisation was obtained by saturating the substrate with copper thiocholine and this tedious step (Reagent 8, p. 164 in Koelle's 1950 paper) was omitted. A later modification including a salting-out technique (Koelle, 1951) gives greater precision of staining without altering the essential basis of the method. The main findings with the 1950 method have been confirmed with the 1951 method.

The acetyl thiocholine was synthesised in this laboratory by Mr. A. N. Davison by the method of Renshaw, Diesback, Ziff and Green (1938). The butyryl thiocholine was a gift from Dr. Bergel of Roche Products Ltd.

Inhibitors.

The following inhibitors were used :

mipafox	N N′-di-isopropyl phosphorodiamidic fluoride
paraoxon	diethyl *p*-nitrophenyl phosphate.
TEPP	tetraethyl pyrophosphate.
TIPP	tetra-isopropylpyrophosphate.
DFP	di-isopropyl fluorophosphonate.
iso-OMPA	tetra-isopropyl pyrophosphoramide.
eserine	(physostigmine).
NU 683	the dimethyl carbonate of (2-hydroxy-5 phenyl benzyl)-trimethyl ammonium bromide.
NU 1250	the N-*p*-chlorophenyl-N-methyl carbonate of *m*-hydroxy-phenyl trimethyl ammonium bromide.
284.c.51	1-5-bis (allyl dimethylammonium phenyl) pentane-3-ene dibromide.

With the irreversible inhibitors, paraoxon, TEPP, DFP, TIPP and iso-OMPA the effect on histochemical staining was studied by incubating sections without substrate at 37° in a series of dilutions of the inhibitor for exactly 30 min., removing and washing in three lots of 0·9 per cent NaCl and then transferring to the substrate used for the staining method. With the reversible inhibitors, eserine, NU 683, NU 1250 and 284.c.51, the sections were first placed in a solution of the inhibitor for 30 min. and then transferred to the substrate which also contained the inhibitor at the same concentration.

Warburg Experiments.

The cholinesterase activity was determined by the method of Nachmansohn and Rothenberg (1945) and the technical details were exactly those described by Aldridge (1950). Myristoyl choline was used at a final concentration of 0·01 M.

Defibrinated sheep red cells that had been stored at 4° were washed 3 times in isotonic saline and re-suspended in buffer to their original volume, immediately before use. Diaphragms were dissected whole from rats, weighed, homogenised in buffer and made up to a volume of 5 ml. or 10 ml. Aliquot parts of 1–5 ml. were taken for cholinesterase determinations. The brain of the rat excluding the cerebellum and medulla was also homogenised in buffer and made up to a final volume of 10 ml. Rat red cells obtained by cardiac puncture were citrated and treated similarly to sheep red cells.

Stroma was prepared by following the first part of the method given by Mounter and Whittaker (1950), 500 ml. of packed red cells from defibrinated sheep blood being lysed with 1,500 ml. of distilled water. The pH was adjusted to 6·5 with 0·1 N HCl. The preparation was centrifuged to collect the stroma and this was washed three times with distilled water. About 100 ml. of stroma was obtained in the form of a thin smooth paste that could still be pipetted and washed into reaction flasks. A purified preparation of specific cholinesterase from rat brain was made by the method of Ord and Thompson (1951).

Chemical Determination of Esterase Activity.

The method was a modification of that of Nachlas and Seligman (1949*b*). Fast blue B (I.C.I.) was used as the coupling agent. In experiments with paraoxon and DFP, 1 ml. of a 1 in 5 dilution of erythrocyte stroma was mixed in a 50 ml. tube with 1 ml. of an aqueous solution of the inhibitor to give the required final concentration of inhibitor, and the mixture was kept at 37° in a water bath. After 30 min. 6 ml. of the buffered solution of β-naphthyl acetate (as described by Nachlas and Seligman, 1949*b*) was added and was followed 16 min. later by 1 ml. of a freshly prepared solution of Fast blue B (2 mg./ml.). The colour was allowed to develop for 4 min. and then 1 ml. of 50 per cent trichloracetic acid was added and the tubes were removed from the water bath. The colour was extracted with 10 ml. of ethyl acetate, the solvent being separated by centrifuging at 1,000 r.p.m. for 5 min. The colour intensity was measured against a reagent blank in a Unicam spectrophotometer at a waveband of 535 Å.

As eserine is a competitive inhibitor and rapidly reaches an equilibrium, in experiments with eserine, the stroma, eserine and β-naphthyl acetate were added simultaneously. As eserine forms a coloured complex with the coupling agent Fast blue B it was necessary to control each concentration of eserine with a similar tube containing identical reagents with the exception of the stroma.

RESULTS.

Gomori method for cholinesterase.

The effect of inhibitors on staining by the Gomori method is shown in the Table. The concentration necessary to reduce the staining to about half intensity is compared with the concentrations necessary to produce 50 per cent inhibition of specific and of non-specific cholinesterase *in vitro*. The figures for 50 per cent inhibition are from many sources and some of the figures have been interpolated from data in the papers quoted. The results show that the staining is reduced

by concentrations of the various inhibitors very similar to the concentrations necessary to produce 50 per cent inhibition of specific cholinesterase. In particular the selective inhibitors of specific cholinesterase, NU 1250 (Hawkins and Mendel, 1949) and 284.c.51 (Austen and Berry, 1953) inhibit staining at very low concentrations. On the other hand the selective inhibitors of non-specific cholinesterase, NU 683 (Hawkins and Gunter, 1946), DFP (Ord and Thompson, 1950), iso-OMPA and mipafox (Aldridge, 1953*b*) prevent staining only when present at high concentrations relative to those required to produce 50 per cent inhibition of non-specific

TABLE.—*Comparison of the Concentrations of Anti-cholinesterases that will reduce Histochemical Staining to Half with the Concentrations producing 50 per cent Inhibition of Specific and Non-Specific Cholinesterase (determined by Warburg Technique).*

Inhibitor.	Molar concentration of inhibitor reducing intensity of staining to half. Method and substrate.				Molar concentration of inhibitor producing 50 per cent inhibition of enzyme.	
	Gomori	Nachlas and Seligman	Koelle.			
	Myristoyl choline.	β-naphthyl acetate.	Acetyl thio-choline.	Butyryl thio-choline.	Specific cholinesterase.	Non-specific cholinesterase.
TEPP	3×10^{-9}	10^{-8}	10^{-8}	10^{-9}	6×10^{-9} (*a*)	$8{\cdot}6 \times 10^{-10}$ (*h*)
Paraoxon	3×10^{-8}	3×10^{-8}	10^{-7}	3×10^{-8}	$2{\cdot}1 \times 10^{-8}$ (*b*)	$6{\cdot}5 \times 10^{-8}$ (*i*)
NU 683	3×10^{-8}	3×10^{-8}	10^{-7}	3×10^{-9}	10^{-8} (*c*)	2×10^{-9} (*c*)
DFP	10^{-7}	3×10^{-7}	10^{-6}	3×10^{-8}	$2{\cdot}7 \times 10^{-7}$ (*d*)	$8{\cdot}0 \times 10^{-9}$ (*d*)
Iso-OMPA	10^{-3}	10^{-3}	10^{-4}	3×10^{-7}	$3{\cdot}1 \times 10^{-3}$ (*e*)	$3{\cdot}3 \times 10^{-7}$ (*e*)
Mipafox	10^{-4}	10^{-4}	10^{-4}	3×10^{-7}	$4{\cdot}5 \times 10^{-5}$ (*e*)	$1{\cdot}7 \times 10^{-7}$ (*e*)
NU 1250	3×10^{-9}	10^{-8}	10^{-8}	3×10^{-5}	3×10^{-9} (*f*)	$6{\cdot}0 \times 10^{-5}$ (*f*)
284.c.51	3×10^{-6}	10^{-6}	10^{-6}	10^{-3}	5×10^{-6} (*g*)	10^{-3} (*g*)
Eserine	3×10^{-6}	10^{-7}	10^{-6}	3×10^{-7}	$1{\cdot}4 \times 10^{-6}$ (*b*)	4×10^{-8} (*j*)

Data for 50 per cent inhibition values from (*a*) Burgen, 1949; (*b*) Aldridge, 1950; (*c*) Hawkins and Gunter, 1946; (*d*) Ord and Thompson, 1950; (*e*) Aldridge, 1953*b*; (*f*) Hawkins and Mendel, 1949; (*g*) Austin and Berry, 1953; (*h*) Brauer, 1948; (*i*) Aldridge, personal communication, 1953; (*j*) Mackworth and Webb, 1948.

cholinesterase. Eserine is used as the only reliable method for separating cholinesterase from ali-esterases, cholinesterases being almost entirely inhibited by a molar concentration of 10^{-5}. At this concentration of eserine, staining is completely inhibited and so the staining cannot be attributed to the activity of an ali-esterase. The histochemical findings are consistent and suggest that the enzyme at the myoneural junction that hydrolyses myristoyl choline is specific cholinesterase.

In view of the histochemical findings it seems important to re-examine whether preparations of specific cholinesterase can hydrolyse myristoyl choline. Estimations of cholinesterase activity by the Warburg method show that appreciable hydrolysis of myristoyl choline is produced by highly active preparations of specific cholinesterase such as rat brain and sheep erythrocytes. There is the possibility, however, that this hydrolysis might be due to a second enzyme such as non-specific cholinesterase or an ali-esterase present as an impurity. This

point has been examined by determining the sensitivity to various inhibitors of the enzymic hydrolysis of acetyl choline and myristoyl choline by sheep erythrocyte stroma. If the two substrates behave similarly with two inhibitors, eserine and DFP, it would then be probable that the same enzyme hydrolyses the two substrates. The inhibition curves shown in Fig. 1 were obtained. With eserine, although the slopes of the inhibition curves are similar, there is, at all concentrations of inhibitor greater sensivitity to eserine inhibition when myristoyl choline is used than when acetyl choline is the substrate. This difference may be due to the fact that acetyl choline has a greater affinity than myristoyl choline for cholinesterase as judged by rates of hydrolysis. A reversible and competitive

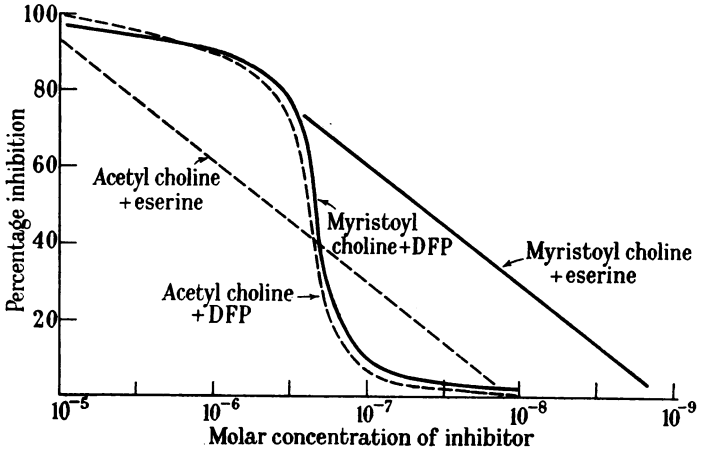


FIG. 1.—The effect of varying concentrations of the inhibitors eserine and DFP on the hydrolysis of myristoyl choline and acetyl choline by sheep erythrocyte stroma.

inhibitor such as eserine might be expected to compete for the enzyme more successfully against myristoyl choline than against acetyl choline. With DFP the inhibition curves are very similar for the two substrates.

Nachlas and Seligman method for esterase.

When fresh frozen sections are immersed in the Nachlas and Seligman substrate the muscle fibres stain uniformly crimson and the line of myoneural junctions stain purple. In paraffin sections of acetone-fixed tissues the general crimson staining persists but the localised purple staining at the myoneural junction is lost. With β-naphthyl acetate as substrate the staining at the myoneural junction takes the form of structureless blobs but with α-naphthyl acetate staining is much more precise (Fig. 4) and resembles closely that obtained with the Gomori method (Fig. 3) and the modified Koelle method (Fig. 5). The effect of various inhibitors of cholinesterase was the same with both α- and β-naphthyl acetate.

The Table shows that the pattern of inhibition of myoneural junction staining by the Nachlas and Seligman method is precisely that found with the Gomori method and corresponds to that of specific cholinesterase. The diffuse crimson staining is much more resistant to inhibition than the purple staining of the myoneural junctions. Thus the diffuse staining is scarcely affected by 10^{-3} M eserine or 10^{-5} M paraoxon. These findings suggest that the purple staining at the myoneural junctions is due to specific cholinesterase and the diffuse crimson staining to A-esterase.

This conclusion implies that specific cholinesterase will hydrolyse α- and β-naphthyl acetate and this point was examined by using the biochemical method of Nachlas and Seligman (1949*b*). With α-naphthyl acetate, the purple colour

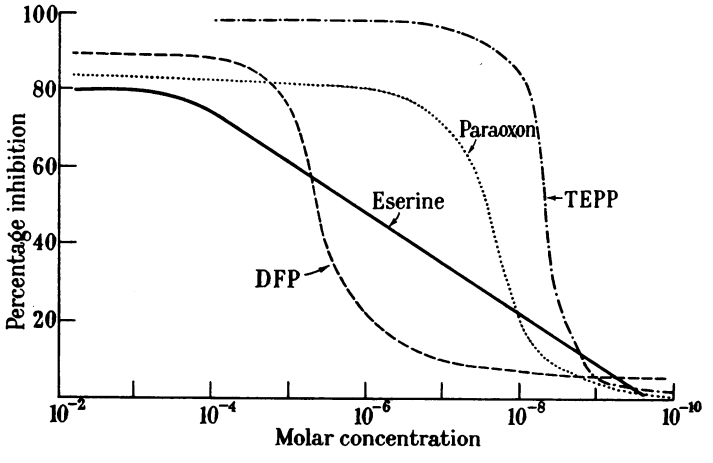


FIG. 2.—The effect of varying concentrations of the inhibitors eserine, DFP, TEPP and paraoxon on the hydrolysis of β-naphthyl acetate by rat brain cholinesterase. These inhibition curves are characteristic of specific cholinesterase : 50 per cent inhibition values are given by the following molar concentrations—eserine 10^{-6}, DFP 5×10^{6}, TEPP 7×10^{9}, paraoxon 4×10^{-8} (*cf.* column 5, Table).

that is formed absorbs strongly on to tissue fragments and is difficult to extract. For this reason β-naphthyl acetate was used in most of these experiments, but enough were done with α-naphthyl acetate to show that the results described for β-naphthyl acetate apply equally to α-naphthyl acetate.

Active but necessarily crude preparations of specific cholinesterase such as rat brain and diaphragm hydrolyse β-naphthyl acetate at rates similar to acetyl choline. Sheep erythrocyte stroma was found to contain an active and eserine-resistant ali-esterase that hydrolysed β-naphthyl acetate, interfering with the detection of any hydrolysis of this substrate by cholinesterase. Partially purified specific cholinesterase from rat brain prepared by the method of Ord and Thompson (1951) was free from non-specific cholinesterase and contained very little of either the acetate- or the butyrate-splitting ali-esterases (the A and B esterases differentiated by Aldridge, 1953*a*), while still retaining much of its specific cholin-

esterase activity. The effects of the inhibitors eserine, DFP, TEPP and paraoxon are shown in Fig. 2. About 80 per cent of the hydrolyses of β-naphthyl acetate is inhibited by each of the compounds at a concentration characteristic of its effect on specific cholinesterase (compare values of 50 per cent inhibition given in the Table). A small amount of the hydrolysis (about 20 per cent) is resistant to the effects of eserine, DFP and paraoxon and can probably be attributed to the small amount of A-esterase known to be present in the enzyme preparation.

Gomori method for lipase (esterase).

With fresh frozen sections, incubated for periods up to 8 hr., diffuse brown staining of muscle fibres was seen, but there was no focal staining at the myoneural junctions. The same results were obtained with acetone-fixed paraffin sections incubated in the substrate for 18 hr.

Koelle method for cholinesterase.

When acetyl thiocholine is used as a substrate the series of inhibitors produces a pattern of inhibition characteristic of their effect on specific cholinesterase (Table).

With butyryl thiocholine as substrate the results are very different. If the incubation time in the substrate is 30 min. as described in Koelle's (1950) paper no staining of the myoneural junctions is seen. If the incubation time is increased to 4 hr. clearly defined staining of the myoneural junctions is obtained (Fig. 6) and this staining is similar to that obtained with acetyl thiocholine (Fig. 5). The effect of selective inhibitors on the relatively slow hydrolysis of butyryl thiocholine is given in the fourth column of the Table, and it can be seen that the inhibition pattern is characteristic not of specific cholinesterase, but of non-specific cholinesterase.

DISCUSSION.

In the Gomori histochemical method, the enzyme hydrolysing the substrate myristoyl choline reacts with selective inhibitors in a way characteristic of specific cholinesterase. Active preparations of cholinesterase hydrolyse myristoyl choline at 3–5 per cent of the rate that they hydrolyse acetyl choline. Eserine was used to decide whether the hydrolysis of myristoyl choline by stroma was due to cholinesterase or an ali-esterase. Myristoyl choline hydrolysis by stroma proved to be more sensitive than acetyl choline hydrolysis to eserine inhibition. What-

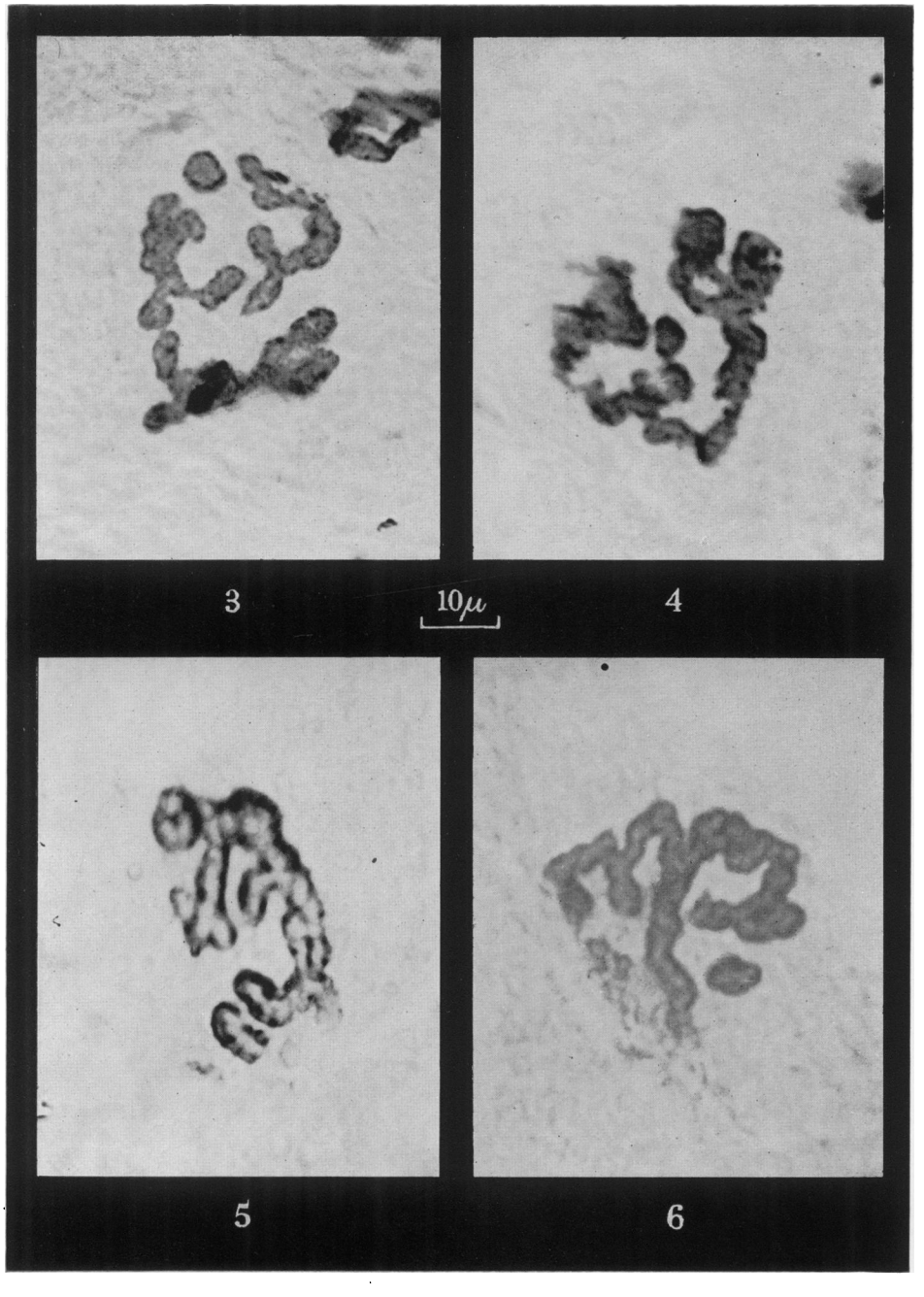
EXPLANATION OF PLATE.

FIG. 3.—Myoneural junction in rat diaphragm. Gomori (1948) method. Substrate—myristoyl choline.

FIG. 4.—Myoneural junction in rat diaphragm. Nachlas and Seligman (1949*a*) method. Substrate—α-naphthyl acetate.

FIG. 5.—Myoneural junction in rat diaphragm. Koelle (1951) method. Substrate—acetyl thiocholine.

FIG. 6.—Myoneural junction in rat diaphragm. Koelle (1951) method. Substrate—butyryl thiocholine.



Denz.

ever the explanation of the displacement of the two lines for eserine inhibition in Fig. 1 it is clear that the enzyme hydrolysing myristoyl choline is very sensitive to eserine inhibition. The curves for DFP are the same for the two substrates. The conclusion reached from these experiments is that specific cholinesterase can hydrolyse myristoyl choline and that the enzyme whose site of action is displayed in Fig. 3 is specific cholinesterase.

The Nachlas and Seligman method for esterases shows the presence of two enzymes in the diaphragm, the first highly active, sharply confined to the myoneural junctions and sensitive to inhibition by eserine and by selective inhibitors of specific cholinesterase and the second spread diffusely over the muscle fibre and resistant to inhibition by eserine. There is other evidence that these two enzymes differ in stability and other properties. If the diaphragm is fixed in cold acetone and paraffin-embedded according to the unmodified method of Nachlas and Seligman (1949*a*) the diffuse staining of the muscle fibres persists, but the myoneural staining is lost. The Gomori (1945) method for lipase shows up the sites of activity of ali-esterases (Nachlas and Seligman, 1949*a*) but there is no evidence that the substrates used in this method, the synthetic sorbitan esters of the higher fatty acids, can be hydrolysed by cholinesterase. With the Gomori method for lipase only diffuse staining of the muscles is observed without any evidence of activity of the myoneural junctions. These findings in conjunction with the effects of inhibitors on the staining by the modified Nachlas and Seligman method as summarised in the Table suggest that the enzyme at the myoneural junction that hydrolyses β-naphthyl acetate is specific cholinesterase and that the enzyme spread diffusely over the muscle fibre is an ali-esterase.

Evidence has been produced to show that specific cholinesterase obtained from rat brain will hydrolyse β-naphthyl acetate. This finding is in agreement with the conclusions of others that specific cholinesterase will hydrolyse a wide range or aromatic acetates (Whittaker, 1951). These observations confirm the histochemical finding that the staining at the myoneural junctions with the Nachlas and Seligman method (Fig. 4) is due to the activity of specific cholinesterase.

As the staining at the myoneural junctions by the Gomori method and the Nachlas and Seligman method can be accounted for by the activity of specific cholinesterase these methods do not disclose the presence of a second enzyme at the myoneural junction. On the other hand, the observation that butyryl thiocholine is hydrolysed at the myoneural junctions is itself suggestive that there is some non-specific cholinesterase activity since Koelle (1950) has shown that specific cholinesterase has little if any effect on butyryl thiocholine. Further, Davison (1953) has shown by using selective substrates and inhibitors that the whole diaphragm of the rat contains an appreciable amount of non-specific cholinesterase. The Gomori method and the Nachlas and Seligman method would not show up the presence of the relatively small activity of non-specific cholinesterase in the presence of considerable specific cholinesterase activity. An examination of the inhibition pattern of the enzyme hydrolysing butyryl thiocholine (Table) shows this to be characteristic of non-specific cholinesterase. An indication of the relative inactivity of the non-specific cholinesterase at the myoneural junction is obtained from the prolonged incubation necessary to obtain histochemical evidence of the activity of the enzyme. In tissues rich in non-specific cholinesterase such as salivary gland and ileum, 30 minutes is adequate for clear histochemical staining. At the myoneural junction staining is still somewhat pale

after the sections have been incubated with butyryl choline 8 times as long as the normal 30 minutes. This suggests that the non-specific cholinesterase at the myoneural junction posseses only a fraction of the hydrolytic activity shown by specific cholinesterase.

The provisional picture of the arrangement of these enzymes in the diaphragm can be drawn. Spread diffusely through the muscle there is an ali-esterase without any focal concentration. At the myoneural junction there are two enzymes sharply confined to the synaptic gutter. The specific cholinesterase is highly active and its site of action is displayed by the Koelle method for cholinesterase when acetyl thiocholine is used as a substrate and by the Gomori method for cholinesterase and by the Nachlas and Seligman method for esterase. The activity of the specific cholinesterase is so much greater than the activity of the non-specific cholinesterase that the latter is hidden with these methods. It has needed the increase in selectivity produced by the additive effects of a selective substrate, prolonged incubation and selective inhibitors to bring to light the non-specific cholinesterase at the myoneural junction.

SUMMARY.

Histochemical staining of the myoneural junctions of the rat diaphragm by the methods of Gomori, of Nachlas and Seligman, and of Koelle, when acetylthiocholine is the substrate, is due to the activity of specific cholinesterase.

Staining by the Koelle method with butyryl thiocholine as substrate is due to non-specific cholinesterase.

Specific cholinesterase is the main enzyme in the synaptic gutter at the myoneural junction, with a small amount of non-specific cholinesterase. An ali-esterase is spread diffusely throughout the muscle fibres without focal concentration at the myoneural junction.

REFERENCES.

ADAMS, D. H., AND WHITTAKER, V. P.—(1948) *Biochem. J.*, **43**, xiv.—(1949) *Biochem. biophys. Acta*, **3**, 358.

ALDRIDGE, W. N.—(1950) *Biochem. J.*, **46**, 451.—(1953*a*) *Ibid.*, **53**, 110.—(1953*b*) *Ibid.*, **53**, 62.

AUSTIN, L., AND BERRY, W. K.—(1953) *Ibid.*, **53**, ix.

BARRNETT, R. J., AND SELIGMAN, A. M.—(1951) *Science*, **114**, 579.

BRAUER, R. W.—(1948) *J. Pharmacol.*, **92**, 162.

BURGEN, A. S. V.—(1949) *Brit. J. Pharmacol.*, **4**, 219.

DAVISON, A. N.—(1953) *Biochem. J.*, in press.

GOMORI, G.—(1945) *Proc. Soc. exp. Biol., N.Y.*, **58**, 362.—(1948) *Ibid.*, **68**, 354.—(1950) *J. Lab. clin. Med.*, **35**, 802.

HAWKINS, R. D., AND GUNTER, J. M.—(1946) *Biochem. J.*, **40**, 192.

Idem AND MENDEL, B.—(1949) *Ibid.*, **44**, 260.

KOELLE, G. B.—(1950) *J. Pharmacol.*, **100**, 158.—(1951) *Ibid.*, **103**, 153.

Idem AND FRIEDENWALD, J. S.—(1949) *Proc. Soc. exp. Biol., N.Y.*, **70**, 617.

MACKWORTH, J. F., AND WEBB, E. C.—(1948) *Biochem. J.*, **42**, 91.

MOUNTER, L. A., AND WHITTAKER, V. P.—(1950) *Ibid.*, **47**, 525.

NACHLAS, M. M., AND SELIGMAN, A. M.—(1949*a*) *J. nat. Cancer Inst.*, **9**, 415.—(1949*b*) *J. biol. Chem.*, **181**, 343.
NACHMANSOHN, D., AND ROTHENBERG, M. A.—(1945) *Ibid.*, **158**, 653.
ORD, M. G., AND THOMPSON, R. H. S.—(1950) *Biochem. J.*, **46**, 346.—(1951) *Ibid.*, **49**, 191.
RAVIN, H. A., TSOU, K. G., AND SELIGMAN, A. M.—(1951) *J. biol. Chem.*, **191**, 843.
RENSHAW, R. R., DREISBACH, P. F., ZIFF, M., AND GREEN, D.—(1938) *J. Amer. chem. Soc.*, **60**, 1765.
RICHTER, D., AND CROFT, P. G.—(1942) *Biochem. J.*, **36**, 746.
WHITTAKER, V. P.—(1951) *Physiol. Rev.*, **31**, 312.