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SOURCES OF ERROR IN RELATING ELECTRICAL AND ACETYLCHOLINESTERASE ACTIVITY*

W.-D. DETTBARN AND P. ROSENBERG†

Departments of Neurology and Biochemistry,
College of Physicians and Surgeons, Columbia University,
New York, N.Y., U.S.A.

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Abstract—The desheathed vagus nerve bundle of the rabbit presents no noticeable barrier to the penetration of acetyl-β-methylcholine (MeCh), which is the specific substrate of acetylcholinesterase (AChE). An attempt was therefore made to determine the minimum level of AChE activity required for electrical activity, by exposing the intact preparation to the organophosphate inhibitor, diisopropylfluorophosphate (DFP).

Conduction appeared to be completely blocked by 5×10^{-2} M DFP but not by 5×10^{-4} M DFP. With MeCh as substrate, the AChE activities of the intact nerve at the two DFP concentrations above were not significantly different. Intact nerves exposed to 5×10^{-4} M and 5×10^{-6} M DFP had higher AChE activities than nerves homogenized in these concentrations of DFP. Vagus nerves from rabbits poisoned *in vivo* with DFP continued to conduct and had significantly higher AChE activity than nerves treated with 5×10^{-2} M DFP *in vitro* which were not conducting.

The bundle of nerves used is composed of about 23,000 fibers, and we can only measure the average enzyme activity. The activity in single fibres after an inhibitor may vary greatly from the initial value to zero. Under these circumstances it is obviously impossible to evaluate the critical level of enzyme activity required for conduction. The importance of this difficulty in attempting to correlate electrical and enzyme activity in multifiber preparations has not been adequately appreciated in the past. It is apparent that, for an unequivocal estimate of the critical level of acetylcholinesterase activity, use of a single fiber preparation is essential.

THE MINIMUM level of AChE in nerve fibers required for unimpaired electrical activity may be important for the understanding of the role of acetylcholine (ACh) in axonal conduction.[1, 2] The evaluation of this level may in principle be obtained by the use of specific and powerful inhibitors of the enzyme. Unfortunately, accurate measurements of the enzyme activity after exposure of the fibers to inhibitors offer many problems and difficulties. In some studies the enzyme activity was determined in homogenized tissue with reversible inhibitors (e.g. Cantoni and Loewi;[3] Keynes and Martins Ferreira[4]). This procedure is inadequate because a large fraction of the inhibitor is concentrated in the extracellular space. If the preparation is washed, both extra- and intracellular fractions of the inhibitor are removed and full activity is restored; without washing, the huge excess in the extracellular space leads to complete inhibition of the intracellular enzyme during the process of homogenization. In contrast, use of irreversible inhibitors may provide pertinent information concerning

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the minimum level of AChE necessary for conduction. However, the removal of the excess extracellular fraction of the inhibitor is extremely difficult and offers many problems. In the best cases there remains always some doubt as to whether complete removal has been obtained.

Procedures in which the enzyme activity is determined on intact axons are preferable and less equivocal; they permit direct measurements of the relationship between the electrical and enzyme activity. Both reversible and irreversible inhibitors can be used. The method has, however, other limitations. It requires the use of a substrate which penetrates readily into the cell interior and thereby provides a satisfactory measure of the total enzyme activity. In two preparations to which this procedure was applied[5, 6]—*i.e.* axons of the spider crab and the squid giant axon—acetylcholine could not be used as substrate since it penetrated poorly. Dimethylaminoethyl acetate (DMAEA) appeared suitable, but it is a poorer substrate than ACh, and less specific for AChE than is MeCh. The rate of penetration is also not entirely satisfactory. Nevertheless, this substrate permitted an estimate of the percentage of the remaining activity at the time when block of conduction occurred. The results with these intact fibers indicated that electrical activity fails when cholinesterase (ChE) falls below 20 per cent of its initial value, a surprisingly good agreement with data obtained previously with homogenized tissue.

Armett and Ritchie[7] recently reported that electrical activity of nonmyelinated, C, fibers of the desheathed vagus nerve of rabbit is affected by externally applied ACh. This indicates that any structural barriers that might exist in this preparation are not great enough to prevent ACh from reaching the conducting membrane. Thus, ACh may be able to assay the intracellular AChE in the intact fibers. It was therefore thought that this preparation might be particularly suitable for relating electrical and enzyme activity in intact axons.

Investigations using the desheathed vagus nerve were initiated, and the results are described in this paper. A new complication was unexpectedly encountered with respect to interpretation. This difficulty is presented and discussed in this paper since it appears of general interest and is pertinent for the evaluation of previous studies.

METHODS

Rabbits were anesthetized with 1·6 g/kg of urethane, and 60 to 70 mm of cervical vagus nerves were excised and, under microscopic control, desheathed.

All recordings of electrical activity in this investigation were made by suspending the nerve on five external electrodes (two recording, two stimulating and one ground). A Grass P6 d.c. preamplifier and S4 stimulator were used. Action potentials were viewed on a Tektronix cathode-ray oscilloscope. Nerves were checked only for the presence or absence of an action potential. Because of the relatively crude method of recording electrical activity no attempt was made to quantitate changes in spike height produced by DFP (see below). All axons were tested after desheathing and found to be conducting.

AChE activity was determined by the Hestrin colorimetric technique.[8] ACh or MeCh bromide was used as substrate. For the enzyme measurements freshly prepared Locke's solution was buffered with 100 mM Tris, adjusted to pH 7·6. The solution had the following millimolar composition: NaCl 154, KCl 5·5, $CaCl_2$ 2·2, and dextrose

5·0. During dissection and for the measurement of electrical activity 1 mM Tris buffer was used.

Axons were shaken with the selected concentration of DFP for 30 min, rinsed 15 min in Locke's solution, and electrical activity was checked. The nerves were preincubated for 1 hr with shaking in a relatively large volume of the desired concentration of MeCh. Since the amount of tissue compared with volume of solution was large, dilution of substrate would result if axons were added to the assay solution without preincubation. After preincubation the nerves were cut in half, placed in the final incubation media, and shaken. Two or three nerves with a total weight of 40 to 80 mg were placed in each incubation vessel of 1-ml volume. Nerves which had not been exposed to DFP served as controls. Absorbance readings were usually taken at 0, 3, and 8 hr.

Five experiments were performed to test whether MeCh is distributed evenly between nerve and external substrate solution. If it were concentrated in nerve tissue we might get erroneous results in our assay. Two DFP-treated axons with very low AChE activity were preincubated in a solution of MeCh for 30 min and then homogenized in 1 ml of MeCh solution of known absorbance similar to the preincubation media. The optical readings were identical before and after homogenization, indicating that MeCh was distributed approximately evenly between nerve and external media. As controls, untreated nerves were homogenized in normal Locke's solution without MeCh.

In another series of experiments nerves were first preincubated with MeCh, then homogenized in 1 ml volume and assayed in the presence of DFP (3 hr from homogenization to end of assay). A few experiments carried out as described above were performed on intact axons in the presence of 5×10^{-6} to 5×10^{-8} M DFP. Electrical activity of the intact axons was checked as previously described after the assay was completed.

In several experiments rabbits were injected intravenously with 300 mg of DFP per animal (about 300 times the LD_{50} dose). They were kept alive with atropine and artificial respiration for 4 hr, after which they were sacrificed. The nerves were excised and checked for electrical activity. AChE activity was determined in the intact axons as previously described for *in vitro* control axons. MeCh 5×10^{-3} M was used as substrate.

RESULTS

The hydrolysis of MeCh was compared in intact and homogenized nerves. As shown in Fig. 1 the rate is about the same in both cases. Each of the points is based on three to ten experiments. The hydrolysis of four concentrations of ACh from 5×10^{-3} M to 5×10^{-4} M was tested in duplicate on intact and homogenized nerves. We observed substrate inhibition when concentrations of ACh greater than 5×10^{-3} M were used. There appeared to be some barrier to the penetration of ACh since homogenized nerves had 10 to 35 per cent greater ChE activity than intact nerves. Because of the few experiments performed, however, and the variability in our results this difference may or may not be significant. These experiments were not pursued further because we were interested in using the substrate specific for acetylcholinesterase; i.e. MeCh for which, as noted above, we found no barrier.

Since there seems to be virtually no barrier to the penetration of MeCh and since, in addition, this substrate is specific for AChE it was selected as substrate for the tests

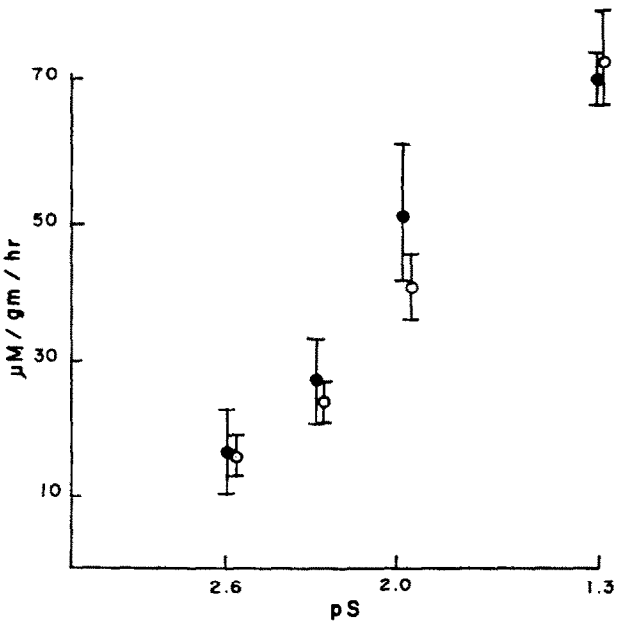

FIG. 1. Acetylcholinesterase activity of intact and homogenized nerve with acetyl-β-methylcholine as substrate. Homogenized nerve (●), intact nerve (○); pS = log of 1 over the molar concentration of substrate. Vertical bars indicate ±1 standard error.

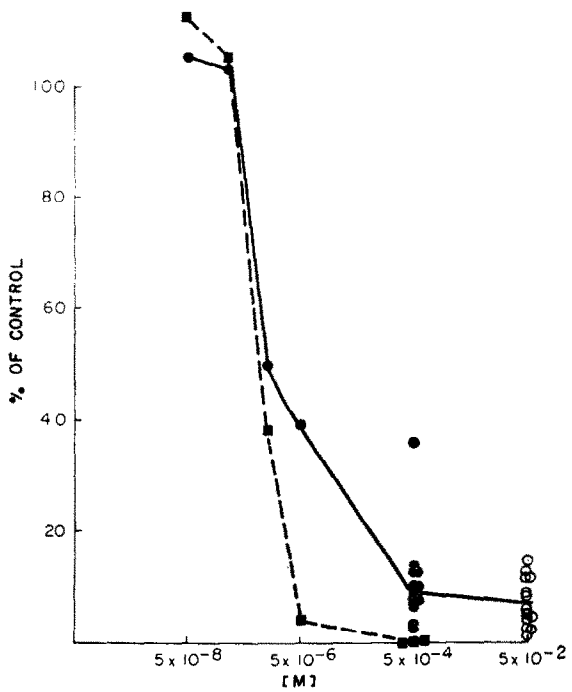

FIG. 2. Comparison of acetylcholinesterase activity in homogenized (■- - -■) and intact (●—●) nerves exposed to various concentration of diisopropylfluorophosphate. Conduction was blocked in all nerves exposed to 5×10^{-2} M DFP (○) but was not blocked in any of the other concentrations.

which were the aim of this paper. Intact and homogenized nerves were exposed to various concentrations of DFP, and AChE activity measured (Fig. 2). The concentration of MeCh was either 5×10^{-2} or 5×10^{-3} M. Since the *d,l* form was used and only one isomer is hydrolyzable[9, 10] the actual substrate concentration was one-half. There was no apparent difference in inhibition by 5×10^{-2} or 5×10^{-4} M DFP at these two substrate concentrations. Each point in the figure represents an individual experiment. In all experiments with 5×10^{-2} M DFP, conduction appeared completely blocked, whereas 5×10^{-4} M DFP or lower concentrations did not block conduction. When, after the exposure to DFP, the fibers were incubated with MeCh for 8 hr, no change as to the presence or absence of an action potential was observed either on the still-conducting or the blocked fibers. The AChE activity (mean $\pm$ standard error) with 5×10^{-2} and 5×10^{-4} M DFP was $7{\cdot}1 \pm 1{\cdot}2$ and $10{\cdot}6 \pm 2{\cdot}2$ per cent of control respectively. The difference is not significant ($P > 0{\cdot}05$). In several experiments with intact axons (not included in Fig. 2) the exposure to 5×10^{-2} and 5×10^{-4} M DFP was 2 hr. These results were not different from those mentioned above where the nerves were exposed to DFP for 30 min.

In nine experiments the AChE activity of vagus nerve from rabbits poisoned *in vivo* was compared with nine experiments in which nerves were treated with 5×10^{-2} M DFP *in vitro*. The former were conducting and the latter not. The enzyme activity (mean $\pm$ standard error) of the first group was $3{\cdot}6 \pm 0{\cdot}3$ whereas the second group had $2{\cdot}4 \pm 0{\cdot}4$ per cent of control activity. The difference is statistically significant ($P < 0{\cdot}05$).

DISCUSSION

The difficulties in the evaluation of the minimum level of AChE required for conduction have been discussed in the past and briefly outlined in the opening paragraphs. The most satisfactory procedure is thought to be the estimation of the enzyme activity in intact fibers exposed to inhibitors. But even this method is not always satisfactory, owing to the presence of structural barriers which prevent the most appropriate substrates from reaching the intracellular enzyme and measuring the rate of hydrolysis under optimal conditions. The desheathed rabbit vagus nerve appeared to be a favorable preparation for determining the relationship between electrical and enzyme activity since MeCh, a specific substrate for AChE readily penetrated into the intact nerve.

In spite of this favorable factor the interpretation of the results encounters a great obstacle. The vagus nerve bundle is composed of about 23,000 fibers.[11] Electrical activity after exposure to DFP was recorded with external electrodes; the method did not permit an exact estimation of the number of fibers conducting. The measured AChE activity is the average of all fibers, therefore one does not know whether the activity present after exposure to DFP represents the activity in each fiber or whether a fraction of the fibers has lower activity and a fraction higher activity than the average. It thus appears impossible to say at what level of enzyme activity electrical activity fails in any particular fiber. Let us assume, for instance that, after exposure to 5×10^{-2} and 5×10^{-4} M DFP, 80 per cent of the fibers had lost all of their activity. The mean values of 7·1 and 10·6 per cent of the initial enzyme activity might then actually represent 35·5 and 53 per cent respectively, when referred to the remaining 20 per cent

of the fibers. If this fraction were conducting, an action potential would be recorded from the nerve bundle, and this might have led to the erroneous conclusion that individual nerve fibers are conducting with only 10·6 per cent of their initial enzyme activity. The uncertainty both as to number of fibers conducting and the actual enzyme activities may also account for the apparent contradictory observations in which nerve fibers were conducting with lower enzyme levels than nerves which were not conducting.

Vagus nerve fibers from rabbits poisoned with 300 times the LD_{50} dose of DFP still are conducting and have a mean enzyme activity that is significantly greater than those in which conduction was blocked by 5×10^{-2} M DFP *in vitro*. However, the exact percentage is subject to the difficulties just discussed.

In homogenized nerve fibers 5×10^{-6} M DFP produced about 96 per cent inhibition of AChE activity, whereas in intact nerve this same concentration inhibited about 60 per cent of the enzyme activity (Fig. 2). The most likely explanation for this difference is the presence of a barrier to DFP in the intact nerve, but other possible explanations are a binding of free DFP to proteins other than AChE in the intact preparation or, alternatively, a greater destruction of DFP in the intact than in the homogenized axons. In these experiments it makes no difference whether the actual values in the intact fibers are about the same in all fibers or close to the initial in some and close to zero in others, since the probable presence of a barrier is confirmed in either case. The presence of barriers was previously demonstrated on the squid giant axon,[12] and their partial reduction was achieved by treatment with venoms.[13, 14]

The difficulties discussed are obviously important for the interpretation of previous data obtained with multifiber preparations. In view of the difficulties described only data obtained with the single axon[6] appear to be reliable.

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REFERENCES

1. D. NACHMANSOHN, *Chemical and Molecular Basis of Nerve Activity*. Academic Press, New York (1959).
2. D. NACHMANSOHN, *Science* **134**, 1962 (1961).
3. G. L. CANTONI and O. LOEWI, *J. Pharmacol. exp. Ther.* **81**, 67 (1944).
4. R. D. KEYNES and H. MARTINS-FERREIRA, *J. Physiol., Lond.* **119**, 315 (1953).
5. I. B. WILSON and M. COHEN, *Biochim. biophys. Acta*, **11**, 147 (1953).
6. W.-D. DETTBARN and F. C. G. HOSKIN, *Biochim. biophys. Acta*. In press.
7. C. J. ARMETT and J. M. RITCHIE, *J. Physiol., Lond.* **155**, 372 (1961).
8. S. HESTRIN, *J. biol. Chem.*, **180**, 249 (1949).
9. D. GLICK, *J. biol. Chem.* **125**, 729 (1938).
10. F. C. G. HOSKIN and G. S. TRICK, *Canad. J. Biochem.* **33**, 963 (1955).
11. D. H. L. EVANS and J. G. MURRAY, *J. Anat., London*, **88**, 320 (1954).
12. E. A. FELD, H. GRUNDFEST, D. NACHMANSOHN and M. A. ROTHENBERG, *J. Neurophysiol.* **11**, **125** (1948).
13. P. ROSENBERG and S. EHRENPREIS, *Biochem. Pharmacol.* **8**, 192 (1961).
14. P. ROSENBERG and T. R. PODLESKI, *J. Pharmacol. exp. Ther.* **137**, 249 (1962).