Biochemical Pharmacology, Vol. 32, No. 1, pp. 151–158, 1983.
Printed in Great Britain.

0006-2952/83/010151-08 $03.00/0

COMPETITIVE INHIBITION BY DIMETHYLSULFOXIDE OF MOLLUSCAN AND VERTEBRATE ACETYLCHOLINESTERASE

JENIFER M. PLUMMER*, MICHAEL J. GREENBERG†, HERMAN K. LEHMAN† and JOHN A. WATTS‡§

Department of Biological Sciences, Florida State University, Tallahassee, FL 32306, and ‡Biology Department, University of North Carolina, Charlotte, NC 28223, U.S.A.

(*Received* 3 *February* 1982; *accepted* 25 *May* 1982)

Abstract—Anticholinesterase-like effects of dimethylsulfoxide (DMSO) were demonstrated on a variety of invertebrate muscles. The excitatory effects of acetylcholine (ACh) on the isolated preparations of the *Geukensia demissa* heart and anterior byssus retractor muscle (ABRM), and of the *Busycon contrarium* radula protractor muscle, were potentiated by DMSO (1–5 μl/ml; 1 μl/ml = 14 mM). The negative chronotropic effects of ACh, but not of 4-ketoamyltrimethylammonium, were potentiated by DMSO (1–5 μl/ml) on the isolated heart of the oyster *Crassostrea virginica*. These four muscles have acetylcholinesterase enzymes of high activity. In contrast, *Mercenaria mercenaria* hearts have weak cholinesterase activity, and the effects of ACh on this isolated myocardium were not potentiated by DMSO (2–20 μl/ml). DMSO (0.1–15 μl/ml) was a competitive inhibitor of both a crude preparation of oyster heart acetylcholinesterase (AChE) (the K_m increased 24-fold with DMSO at 15 μl/ml; the I_{50} was 1.3 μl/ml DMSO when [ACh] = K_m) and a purified *Electrophorus* AChE (the K_m increased 4.5-fold when DMSO was 10 μl/ml; the I_{50} was 10 μl/ml DMSO near [ACh] = K_m). The same doses of DMSO were needed to potentiate the pharmacological effects of ACh on the oyster heart, as to inhibit the AChE of this tissue.

Dimethylsulfoxide (DMSO) is a controversial drug which was used in clinical studies in the early 1960s and has recently received renewed attention. Interest in DMSO stems from its rapid penetration through the skin [1, 2], its cryoprotective properties [3, 4], and its reduction of the pain and swelling associated with arthritis [5] and sports-related injuries [6]. Clinical trials indicate that DMSO is relatively nontoxic [7], though not without side effects.

Brobyn [7] noted that 30–50% of the human subjects in his study complained of dizziness, nausea and sedation during treatment with DMSO. Teratogenic effects and ocular changes may also be associated with this drug (reviewed by David [1]). At the cellular level, DMSO appears to cause dissolution of cytoplasmic microfilaments, thereby blocking cytokinesis and inducing the formation of nuclear actin bundles [8–11]. DMSO also appears to block insulin release [12], inhibit the pigmentation of neural crest cells [13], and depress nerve conduction in frogs [14]. Moreover, DMSO inhibits prostaglandin synthesis [15], which may, in part, account for its analgesic and anti-inflammatory effects, since prostaglandins may be important in inflammatory responses [16]. Finally, and most germane to the present study, DMSO has been reported to inhibit acetylcholinesterase (AChE).

Pharmacological data obtained from a scattering of vertebrate nerve or muscle preparations indicate that DMSO inhibits AChE. The vagal threshold of guinea pig atria is reduced by 6 μl/ml DMSO [17, 18]. Infusion of DMSO into the circulatory system of cats results in sinus bradycardia [19], which may be due to enhanced parasympathetic tone. Shlafer *et al.* [20] found that the negative chronotropic effect of 10 μl/ml DMSO on rabbit atria was blocked by atropine, and they postulated that the effect resulted from the build up of endogenous acetylcholine (ACh) due to AChE inhibition. In chick skeletal muscle, twitches initiated by nerve stimulation or by ACh are augmented with 10–50 μl/ml DMSO, whereas carbachol-induced twitches are not [21]. Carbachol is an ACh agonist which is not hydrolyzed by AChE. In addition, diaphragm muscle exhibits fasciculations in the presence of 30–60 μl/ml DMSO, and the response of stomach smooth muscles to nerve stimulation is increased by 30 μl/ml DMSO [17, 18]. Both excitatory and inhibitory postsynaptic potentials in the central nervous system of *Aplysia* are enhanced by low doses (1 μl/ml) of DMSO, suggesting AChE inhibition; higher doses (10 μl/ml) block the acetylcholine receptor and decrease ion conductance [22].

Cholinesterase inhibition by DMSO has been measured directly in a few systems, but the kinetic conditions of the assay have not been well defined. Thus, the mode of inhibition has not been elucidated. Sams and Carroll [17] reported that 0.78, 7.8, 39 and

* Present address: Department of Physiology, Royal Veterinary College, University of London, London, NW1 0TU, England.

† Present address: C. V. Whitney Laboratory, University of Florida, Route 1, Box 121, St. Augustine, FL 32084, U.S.A.

§ Send reprint requests to: Dr. John A. Watts, Department of Biology, University of North Carolina at Charlotte, UNCC Station, Charlotte, NC 28223, U.S.A.

78 μl/ml DMSO reduce bovine red blood cell butyrylcholinesterase by 3, 16, 44, and 85%. The dose which inhibited 50% of the enzyme activity (I_{50}) was 45 μl/ml. In their preliminary letter, Gandiha and Marshall [21] noted that chick skeletal muscle cholinesterase (ChE) is inhibited 58% with 50 μl/ml DMSO. In rabbit atria, 10–100 μl/ml DMSO inhibits 20–77% of the ChE activity (I_{50} = 60 μl/ml) [20]. In contrast to the ChE inhibition observed in the above studies, Wachtendonk and Neef [23] have reported that the soluble haemolymph AChE of the bivalve mollusc *Mytilus* is stimulated by DMSO.

In this paper, we first describe the pharmacological effects of DMSO on the responses of several invertebrate muscles to ACh; some of the responses are excitatory and some inhibitory. We then demonstrate that DMSO inhibits the AChE activity of crude (molluscan) and purified (vertebrate) enzyme preparations, and analyze the kinetics of the inhibition. Similar pharmacological and enzymatic studies have been performed recently with mammalian heart muscle, and the results obtained in that tissue are analogous to those reported here [24].

MATERIALS AND METHODS

Animals

Specimens of *Crassostrea virginica*, the American oyster, were obtained from a local market in Tallahassee, FL; *Geukensia demissa granosissima*, the southern ribbed mussel, and *Busycon contrarium*, the lightning whelk, were collected, respectively, from marshes and sandbars along the coast of the Gulf of Mexico in Franklin County, FL. *Mercenaria mercenaria*, the quahog, was purchased from the Northeast Marine Specimens Co., Woods Hole, MA.

These animals were maintained in aerated aquaria, in natural seawater (salinity, 30–32 parts per thousand), at 22°. All of the animals were used within 2 weeks of their collection or purchase.

Preparation of isolated hearts and muscles

Mercenaria and *Geukensia* hearts were isolated according to the classical method of Welsh and Taub [25]. Oyster hearts are constructed differently than those of most other bivalves, so *Crassostrea* ventricles were isolated as described by Greenberg [26]. The anterior byssus retractor muscles (ABRM) of *Geukensia* were prepared by the method of Painter [27], and the radula protractor muscles (RPM) of *Busycon* according to the procedure of Hill [28].

The isolated muscles were immersed in aerated, water-jacketed organ baths (5 ml; 16°) and were stretched between a stainless steel hook at the bottom of the bath and a force-displacement transducer (Grass model FT. 03C) placed above. A light spring (k = 300 mg/cm) was interposed between the muscle and the transducer; therefore, the contractions were auxotonic. The mechanical activity was recorded with an ink-writing oscillograph (Grass polygraph).

The bathing medium was natural seawater (30–32 parts per thousand). Drugs were added directly to the medium with a syringe, and all doses are expressed as final concentrations in the bath. Since DMSO is a liquid at room temperature, volumetric doses were administered in the interests of convenience, precision and accuracy. The dose concentrations are presented in the the graphs as μl of pure DMSO per ml of bath fluid, but some values in mM are also given in the text and figure legends. To convert: 1 μl/ml = 14 mM.

The following drugs were used: acetylcholine iodide (ACh) (Sigma Chemical Co., St. Louis, MO); 4-ketoamyltrimethylammonium iodide (4K) (K & K Laboratories, Plainview, NY); and DMSO (Sigma).

Tissue preparation for AChE assay

Pooled oyster hearts in 0.1 M phosphate buffer (five hearts/ml; pH 8.0; 4°) were homogenized by 20 passes in a motor-driven Potter–Elvehjem homogenizer with a Teflon pestle and were then centrifuged at 1000 *g* for 30 min (4°). The supernatant fraction, containing 5.2 ± 0.31 mg protein/ml (mean ± S.E.M., $N = 4$), was assayed for AChE (EC 3.1.1.7) activity. Roop and Greenberg [29] have demonstrated that the kinetics of AChE activity in homogenates and purified preparations from oyster hearts are the same with respect to substrate activation and inhibition by known AChE inhibitors.

For comparison, purified *Electrophorus electricus* (eel) AChE (EC 3.1.1.7) (Worthington Biochemical Corp., Freehold, NJ) was dissolved in 0.1 M phosphate buffer (pH 8.0) to a concentration of 1.81 μg protein/ml, and this preparation was also assayed.

AChE assay

AChE was assayed by a modification of the method of Ellman *et al.* [30]. Paired test tubes contained either 2.8 ml of 0.1 M phosphate buffer (pH 8.0) plus 0.1 ml of oyster enzyme, or 2.89 ml of buffer plus 0.01 ml of eel enzyme. In addition, each tube contained 0.1 ml of 0.1 M 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma), a solution made up in 0.1 M phosphate buffer (pH 7.0). The reaction, carried out at 23° was initiated by the addition of 20 μl of the appropriate acetylthiocholine (AThCh) substrate solution to one of the paired tubes. The other tube was the enzyme blank. We terminated the reactions by adding 40 μl of 1.5×10^{-3} M eserine sulfate (final concentration, 2×10^{-5} M) to both the reaction and blank tubes. Preliminary experiments showed that this concentration of eserine would inhibit 97% of the oyster heart AChE and 99% of the eel AChE. After the reaction had been stopped, substrate was added to blanks, and the absorbance was measured in a Beckman DB-G spectrophotometer at 412 nm wavelength. When DMSO was tested, it was added to both the reaction tubes and the blanks 5 min before the reaction was initiated by the addition of substrate; the brief preliminary incubation with DMSO ensured that full inhibition would develop.

The difference in absorbance between the experimentals and blanks is a function of the number of free SH groups exposed to the hydrolysis of substrate. The thiocholine released reacts with DTNB to produce a yellow product. Extinction units were converted to μmoles of substrate hydrolyzed by comparison with a glutathione standard curve [31]. Protein was determined by the method of Lowry *et al.*

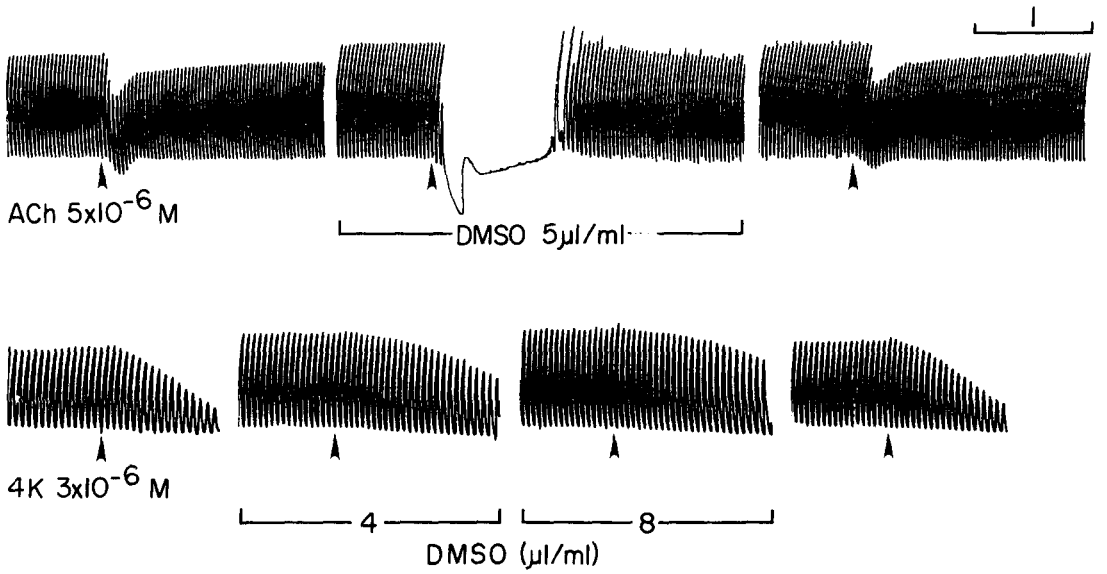

Fig. 1. Reversible effects of DMSO on the responses of the oyster heart to acetylcholine (ACh) and 4-ketoamyltrimethylammonium iodide (4K). DMSO was added 5 min before the agonist; the preparations were washed for at least 5 min between the additions of ACh or 4K. Time: 1 min; 1 µl DMSO/ml = 14 mM.

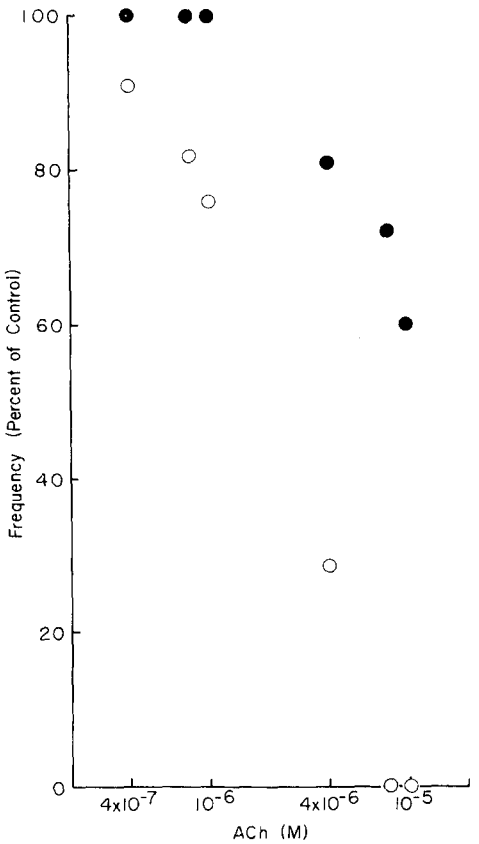

Fig. 2. Effect of DMSO (2 µl/ml) on a typical ACh concentration–response curve from an isolated heart of the oyster *C. virginica*. Ordinate: ratio of the number of beats for 2 min after the addition of ACh to the number of beats for 2 min before the addition, multiplied by 100 (see Greenberg *et al.* [33]). Key: (●) ACh control and (○) ACh + DMSO (2 µl/ml). One µl DMSO/ml = 14 mM.

[32]; a bovine serum albumin standard (Sigma) was used.

Preliminary studies indicated that the enzyme reactions were linear with time and protein concentration under the conditions described above.

Determination of K_m *and* V_{max}

The Michaelis constants K_m and V_{max} were determined from linear regression analysis of double-reciprocal plots by the method of least squares.

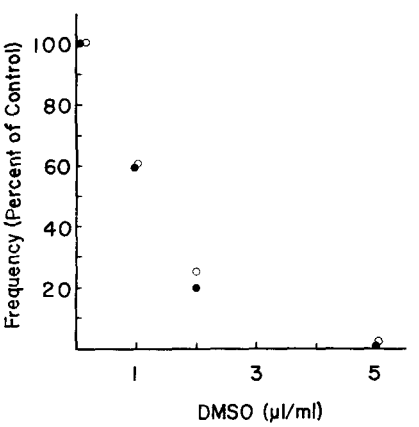

Fig. 3. Concentration–response curve for the potentiation, by DMSO, of ACh inhibition of the isolated heart from the oyster *C. virginica*. ACh test concentration: 4×10^{-6} M. Ordinate: computations as in Fig. 2. Open and closed circles: the results from two preparations are plotted. One µl DMSO/ml = 14 mM.

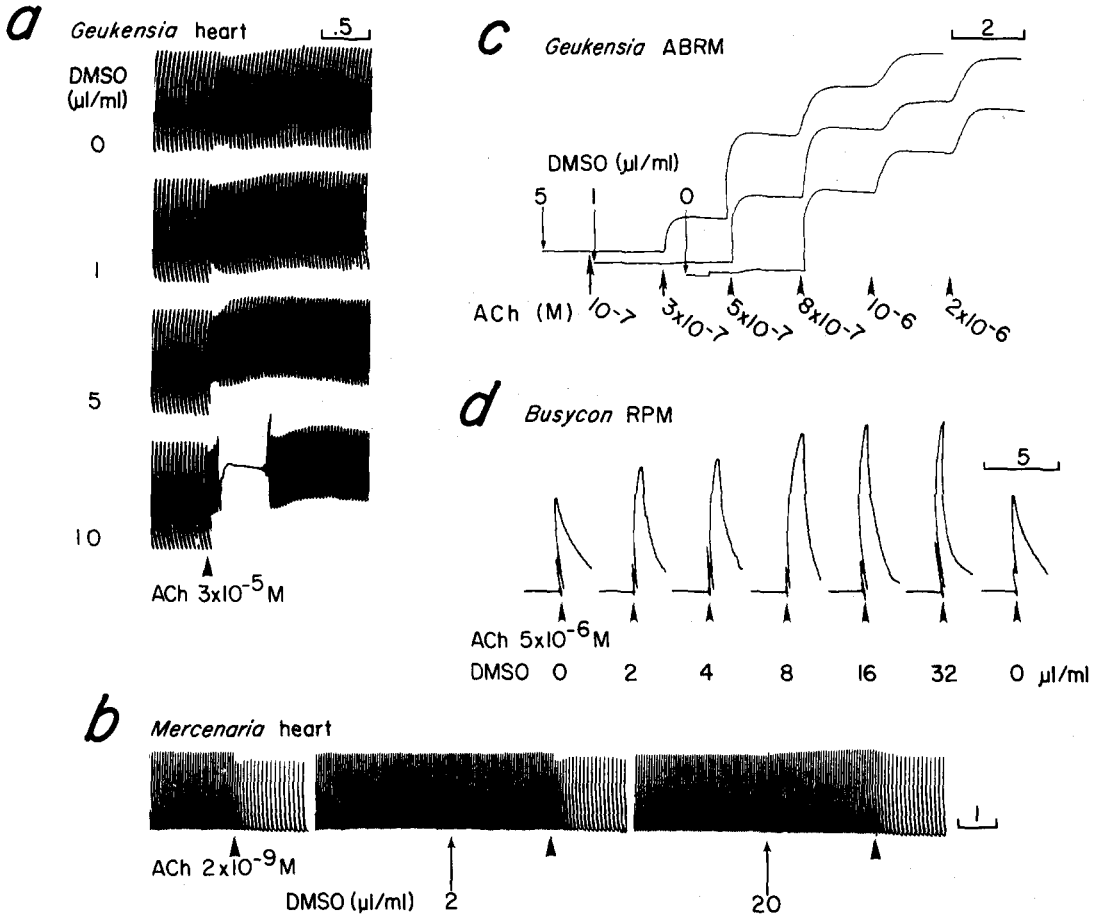

Fig. 4. Effects of DMSO on the responses of some isolated molluscan heart and muscle preparations to ACh. DMSO was given 5 min before ACh was added. (a, b, d) The ACh, or ACh + DMSO, was washed out of the bath for 5 min before the subsequent addition was made. (c) Three cumulative concentration–response recordings produced by a series of ACh additions to the bath, with no intervening washs, to make the concentrations indicated. The muscle was washed for 15 min between each set of additions. The control recording (DMSO = 0 µl/ml) was produced first; DMSO (1 and 5 µl/ml, respectively) was added to the bath 5 min before the next two recordings. Time: 1 min; 1 µl DMSO/ml = 14 mM.

RESULTS

Pharmacology of DMSO

In the concentrations usually used in these experiments (1–5 µl/ml; 14–70 mM), DMSO had no effect on the beat frequency of oyster hearts (N = 20), although, at 5 µl/ml, there were infrequent, small changes in tone and amplitude. At high doses (10–50 µl/ml; 140–700 mM), DMSO had a moderate positive inotropic effect (e.g. Fig. 4b); in some cases this was accompanied by a decrease in frequency.

Effects on cholinergic inhibition of oyster hearts

The threshold of cardioinhibition by ACh was high: 1.8×10^{-6} M ± 0.45×10^{-6} M (mean ± S.E.M., N = 14). The primary effect was negative chronotropy, and even the largest dose tested, 3×10^{-4} M, produced only a transient diastolic arrest. The reduced frequency, measured 2 min after adding ACh, was expressed as a percentage of that of the control beat [33]. The IC_{50}, the concentration producing 50% of the control frequency, was 1.7×10^{-5} M ± 0.37×10^{-5} M.

When DMSO was present in the medium for 1–5 min before the addition of ACh, the inhibition subsequently produced by ACh was potentiated (Fig. 1). ACh dose–response curves generated in the presence of DMSO were shifted to the left of control curves (Fig. 2); with 2 µl/ml (28 mM) DMSO, the ratio of equiactive doses was 4.3 ± 0.38 (mean ± S.E.M., N = 7). The potentiating action of DMSO was dose dependent (Fig. 3); threshold was between 0.5 and 1 µl/ml (7–14 mM).

4-Ketoamyltrimethylammonium iodide (4K), a potent analog of ACh in the oyster heart, is not attacked by AChE. The threshold to 4K is lower than that of ACh on untreated hearts, and its effects are sustained, rather than transient. Moreover, those effects are partially antagonized by anti-AChE agents [33]. DMSO, in a series of concentrations from 4 to 20 µl/ml (56 to 280 mM), never potentiated the action of 4K. Rather, the 4K-induced cardioinhibition was partially blocked by DMSO (Fig. 1), and the antagonism was concentration dependent.

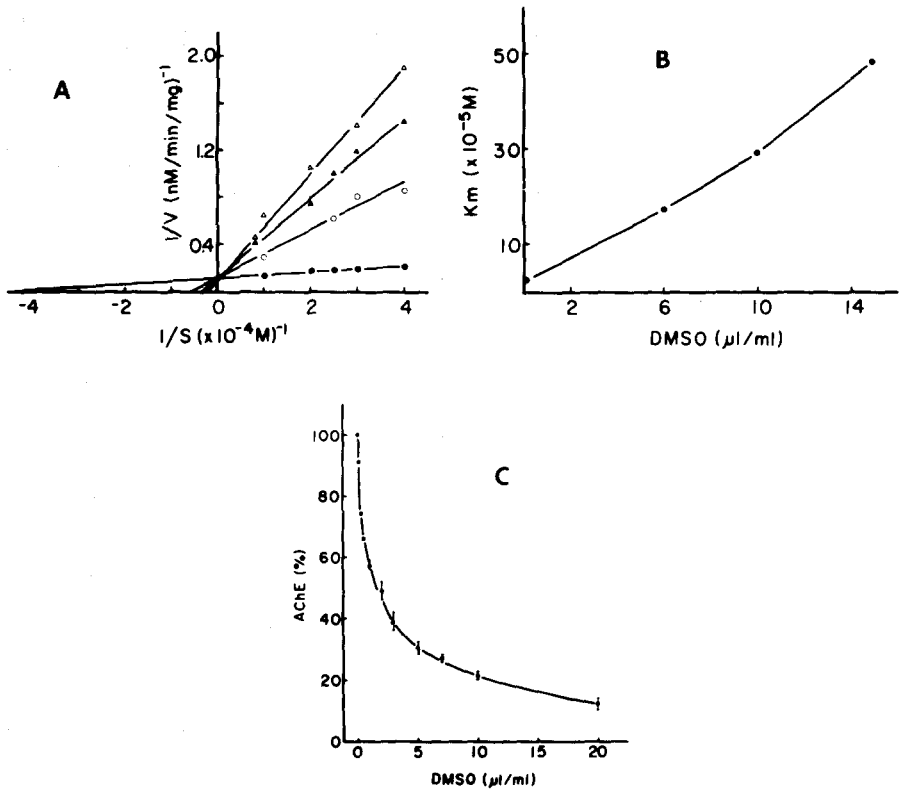

Fig. 5. Effects of DMSO on *C. virginica* (oyster) heart AChE activity. (A) Lineweaver–Burk plot. The reaction mixtures contained 0.5 mg protein. The enzyme was preincubated with DMSO for 5 min prior to the addition of substrate (acetylthiocholine). Reaction temperature: 23°; 1 µl DMSO/ml = 14 mM. Regression equations: control, $y = 0.0253x + 0.1170$, $r^2 = 0.9864$ (●); 6 µl DMSO/ml, $y = 0.2020x + 0.1170$, $r^2 = 0.9791$ (○); 10 µl DMSO/ml, $y = 0.3336x + 0.1137$, $r^2 = 0.9856$ (▲); and 15 µl DMSO/ml, $y = 0.4436x + 0.0091$, $r^2 = 0.9990$ (△). (B) Effect of DMSO on the apparent Michaelis constant (K_m). (C) Effect of DMSO on AChE activity (mean ± S.E.M., N = 3). The substrate concentration (2.5×10^{-5} M) was equal to the apparent K_m. One hundred percent activity was 4.5 ± 0.65 nM per min per mg protein.

Correlative observations of other molluscan hearts and muscles

Hearts of the mussel *Geukensia* differ from those of the oyster in being excited by ACh, but they are similar in that the threshold (3×10^{-7} to 10^{-5} M) and the endogenous AChE activity are high [29, 33, 34]. In addition, the effects of ACh are clearly potentiated by eserine [33, 35]. DMSO, at concentrations as low as 1 µl/ml, potentiated the increase in tone and frequency produced by ACh; the potentiation increased with the concentration of DMSO (Fig. 4a).

The heart beat of the venerid clam *M. mercenaria,* like that of oysters, is reduced in frequency and amplitude by ACh [25]. But clam tissues contain a butyrylcholinesterase, and its activity in the myocardium is very low [29]. Thus, the effects of ACh are poorly potentiated by anticholinesterases [33]. A wide range of DMSO concentrations were tested on *Mercenaria* hearts, but there was no effect on ACh cardioinhibition in this species (Fig. 4b).

The anterior byssus retractor muscle (ABRM) of *G. demissa granosissima* is contracted by ACh, and this effect is augmented by treatment with eserine [27]. ABRMs treated with DMSO (1–5 µl/ml) contracted more forcefully than controls treated with the same concentration of ACh (Fig. 4c). The mean ratio of equiactive concentrations was 5.5 with 1 µl/ml DMSO; the same value was obtained with 10^{-5} M eserine (data not shown) which is within the range (3 to 30-fold potentiation) of eserine effects reported by Painter [27].

The radula protractor muscle (RPM) of the whelk *B. contrarium* appears to receive an excitatory cholinergic innervation [36, 37], and eserine has long been known to potentiate the effects of ACh on that muscle [28]. DMSO potentiated ACh contractures of the RPM, and the effect increased with increasing concentration (Fig. 4d).

Effect of DMSO on AChE activity

Oyster AChE. DMSO was found to be a competitive inhibitor of oyster AChE with respect to substrate acetylthiocholine (Fig. 5A). When DMSO was increased from 0 to 15 µl/ml of the reaction solution (210 mM), the apparent K_m of AChE for acetylthiocholine rose from 2.2×10^{-5} M to 48.6×10^{-5} M (Fig. 5B), a 24-fold increase, whereas V_{max} (8.6 nM per min per mg) remained unchanged.

When acetylthiocholine was present at 2.5×10^{-5} M (near K_m), DMSO caused strong inhibition

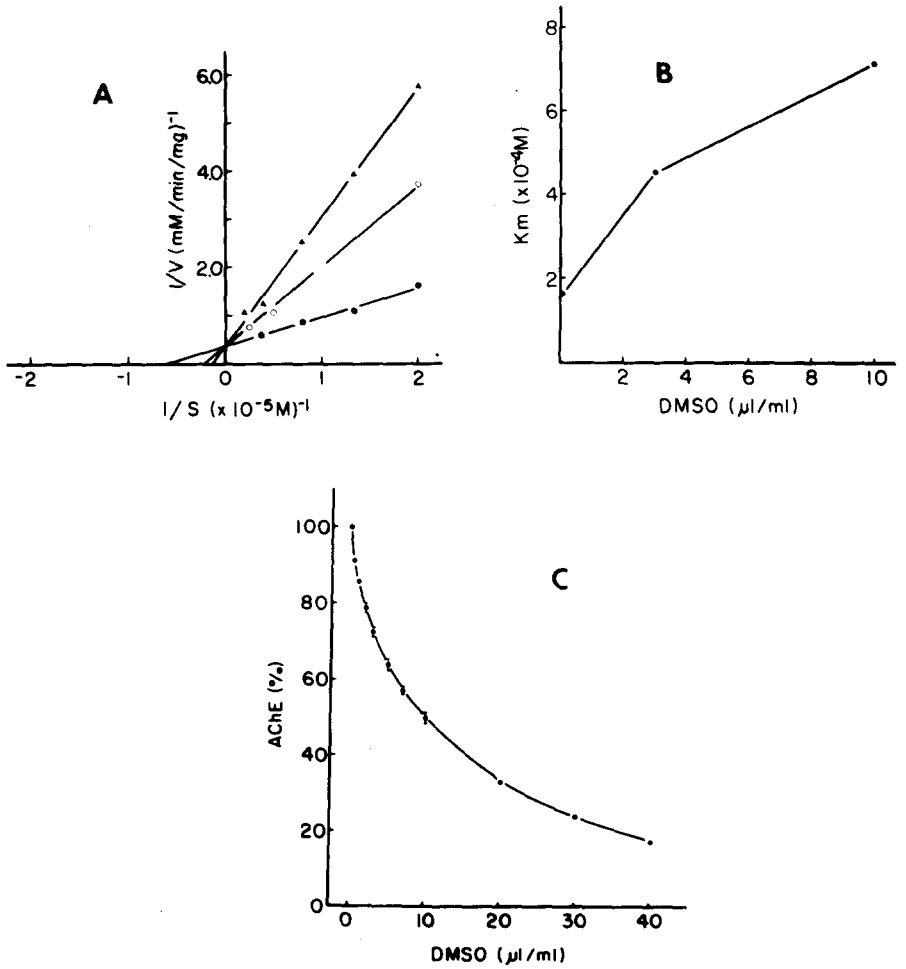

Fig. 6. Effects of DMSO on *E. electricus* (eel) electric organ AChE. (A) Lineweaver–Burk plot. The reaction mixtures contained 18.1 ng protein. The enzyme was preincubated with DMSO for 5 min prior to the addition of substrate (acetylthiocholine). Reaction temperature: 23°, 1 µl DMSO/ml = 14 mM. Regression equations: control, $y = 0.6007x + 0.3682$, $r^2 = 0.9770$ (●); 3 µl DMSO/ml, $y = 1.6730x + 0.3682$, $r^2 = 0.9976$ (○); and 10 µl DMSO/ml, $y = 2.7021x + 0.3769$, $r^2 = 0.9973$ (▲). (B) Effect of DMSO on the apparent Michaelis constant (K_m) of eel AChE. (C) Effect of DMSO on AChE activity (mean ± S.E.M., N = 3). The substrate concentration (5×10^{-4} M) was near K_m. One hundred percent activity was 1.67 ± 0.043 mM per min per mg protein. Points without error bars: the error term is smaller than the symbol.

of the oyster AChE (Fig. 5C). At 1 µl DMSO/ml of reaction, AChE activity was only 57.4% of control activity. This concentration of DMSO also caused marked potentiation of the pharmacological effect of ACh on the intact beating oyster heart (see Fig. 3).

Eel AChE. DMSO was also a competitive inhibitor of the purified eel AChE. When the DMSO concentration was varied from 0 to 10 µl/ml of reaction solution, the apparent K_m for acetylthiocholine increased from 1.6×10^{-4} M to 7.2×10^{-4} M, while the V_{max} (2.72 mM per min per mg) was unchanged (Fig. 6A and B). In the presence of 5×10^{-4} M acetylthiocholine (near K_m), DMSO caused pronounced inhibition of AChE activity (Fig. 6C).

DISCUSSION

The pharmacological data reported here strongly support the notion that DMSO is an AChE inhibitor. The argument is as follows. DMSO potentiated the action of ACh whether that action was excitatory or inhibitory. But DMSO did not potentiate the effect of 4K, a 4-ketoamyl analog of ACh that lacks the ester linkage and is, therefore, not hydrolyzed by AChE. In fact, the muscle responses to 4K were decreased by DMSO, suggesting partial blockade of the cholinergic receptors; this is another well described characteristic of molluscan anti-AChE agents [33]. It has been shown, either biochemically (hearts of *Crassostrea* and *Geukensia*) or pharmacologically (ABRM and RPM), that those preparations in which ACh responses were potentiated by DMSO have moderate to strong AChE activity (references in appropriate sections of the Results). Finally, the *Mercenaria* heart, a preparation sensitive to ACh, but known to lack AChE [29], showed no DMSO potentiation of cholinergic inhibition. These

results confirm earlier studies of vertebrate systems (references in the beginning of the paper).

The pharmacologically founded conclusion that DMSO is an AChE inhibitor is confirmed and extended by our biochemical findings. That is, whether it was tested on crude preparations (oyster homogenate) or purified electric eel AChE, DMSO was a competitive inhibitor of the enzyme. In fact, the chemical structure of DMSO resembles both the choline portion and the carbonyl group of ACh. Thus, the $-S(CH_3)_2$ group of DMSO may bind to the cationic binding site on AChE or, alternatively, the $-S=O$ group may bind to the esteratic site of the enzyme, thereby competing with the substrate ACh. In support of this notion, Krupka [38] found that the activity of eel and red blood cell AChE was inhibited (60–90%) by DMSO (10–60 μl/ml). These assays were carried out with *p*-nitrophenyl acetate, a neutral substrate which binds to the acylation residues of the esteratic site of AChE.

At the apparent K_m concentrations of the substrate, AChE was very sensitive to DMSO inhibition. For example, the AChE of oyster heart homogenates was half-inhibited (I_{50}) with 1.3 μl/ml (18.2 mM) DMSO when acetylthiocholine was used at the apparent K_m concentration. The eel AChE was 50% inhibited at 10 μl/ml DMSO, but the concentration of the substrate was three times K_m during this experiment. The apparent K_m value of an enzyme usually represents a physiological concentration of the substrate [39]. Hence, the doses of DMSO which induced enzyme inhibition under nonsaturating substrate conditions may correlate with the pharmacologically effective concentration of DMSO. In fact, in oyster heart there was a good correlation between the concentrations of DMSO required for AChE inhibition and for potentiation of ACh effects. For instance, the reduction in oyster heart beat frequency induced by 1×10^{-5} M ACh was increased from 60 to 100% by DMSO at 2 μl/ml (see Fig. 2). Under similar conditions, i.e. 2.5×10^{-5} M acetylthiocholine and 2 μl/ml DMSO, oyster heart AChE was inhibited by 56% (see Fig. 5C).

Finally, in contrast to our studies, as well as those on vertebrates, haemolymph AChE of *Mytilus edulis* is stimulated 30–40% by DMSO (20–40 μl/ml) [23]. However, no kinetic data were presented, so the concentration of substrate relative to K_m is unknown. In addition, unlike the enzymes in the present study, the *Mytilus* haemolymph AChE is a soluble enzyme [23].

DMSO in higher doses (80–200 μl/ml) seems to have a direct effect on ion conductances in *Aplysia* CNS [22] and blocks acetylcholine receptors in skeletal muscle [21] and *Aplysia* CNS [22]. But, high concentrations of DMSO also have nonspecific osmotic effects. This notion is supported by the observation that reperfusion of isolated rat hearts with Krebs buffer following DMSO treatment (50–150 μl/ml; 0.7–2.1 M) causes ultrastructural damage [40] and release of cytoplasmic enzymes [41]. In addition, treatment of isolated mammalian hearts with high doses of DMSO results in dose-dependent positive and negative inotropic responses possibly due to changes in cellular hydration [42]. The concentrations of DMSO used in the present experiments do not induce nonspecific ion permeability changes, since DMSO alone does not alter muscle contraction except at very high doses (20 μl/ml).

Acknowledgements—This work was supported in part by the following grants: The Royal Society of England and the Wain Fund of the ARC to J. M. Plummer, NIH HL-09283 to M. J. Greenberg, and the Foundation of UNCC and the State of North Carolina, The N.C. American Heart Association, and the North Carolina United Way to J. A. Watts. This is contribution number 182 from the Tallahassee Sopchoppy and Gulf Coast Marine Biological Association.

REFERENCES

1. N. A. David, *A. Rev. Pharmac.* **12**, 353 (1972).
2. H. H. Szmant, *Ann. N.Y. Acad. Sci.* **243**, 20 (1975).
3. F. G. J. Offerijns, G. E. Freud and H. W. Kritnen, *Nature, Lond.* **222**, 1174 (1969).
4. M. Shlafer and A. M. Karow, *Cryobiology* **8**, 280 (1971).
5. P. Görög and I. B. Kovacs, *Ann. N.Y. Acad. Sci.* **243**, 91 (1975).
6. L. Lazorko, *Phys. Sports Med.* **8**(8), 16 (1980).
7. R. D. Brobyn, *Ann. N.Y. Acad. Sci.* **243**, 497 (1975).
8. J. T. Sibley, P. D. Matthew and E. D. Hanson, *J. Protozool.* **24** (4), 595 (1977).
9. Y. Fukui, *J. Cell Biol.* **86**, 181 (1980).
10. J. W. Sanger, J. Gwinn and J. M. Sanger, *J. exp. Zool.* **213**, 227 (1980).
11. J. W. Sanger, J. M. Sanger, T. E. Kreis and B. M. Jockushch, *Proc. natn. Acad. Sci. U.S.A.* **77** (9), 5268 (1980).
12. D. B. McKay and A. M. Karow, *Res. Commun. Chem. Path. Pharmac.* **30** (1), 15 (1980).
13. J. H. Greenberg and C. Oliver, *Archs Biochem. Biophys.* **204** (1), 1 (1980).
14. W. M. Sams, *Ann. N.Y. Acad. Sci.* **141**, 242 (1967).
15. T. R. La Hann and A. Horita, *Proc. west. Pharmac. Soc.* **18**, 81 (1975).
16. J. Garcia-Rafanell, *Arzneimittel-Forsch.* **29** (4), 630 (1979).
17. W. M. Sams and N. V. Carroll, *Nature, Lond.* **212**, 405 (1966).
18. W. M. Sams, N. V. Carroll and P. L. Crantz, *Proc. Soc. exp. Biol. Med.* **122**, 103 (1966).
19. K. I. Mellville, B. Klingner and H. E. Shister, *Archs int. Pharmacodyn. Thér.* **174**, 277 (1968).
20. M. Shlafer, J. L. Matheny and A. M. Karow, *Archs int. Pharmacodyn. Thér.* **221**, 21 (1976).
21. A. Gandiha and I. G. Marshall, *J. Pharm. Phamac.* **24**, 417 (1972).
22. M. Sawada and M. Sato, *Ann. N.Y. Acad. Sci.* **243** 337 (1975).
23. D. von Wachtendonk and J. Neef, *Z. Naturf.* **33**, 851 (1978).
24. J. A. Watts and R. P. Hoogmoed, *Fedn Proc.* **41** (5), 1605 (1982).
25. J. H. Welsh and R. Taub, *Biol. Bull. mar. biol. Lab., Woods Hole* **95**, 346 (1948).
26. M. J. Greenberg, *Comp. Biochem. Physiol.* **14**, 513 (1965).
27. S. D. Painter, *J. comp. physiol.*, in press.
28. R. B. Hill, *Biol. Bull. mar. biol. Lab., Woods Hole* **115**, 471 (1958).
29. T. Roop and M. J. Greenberg, *J. exp. Zool.* **198** (2), 121 (1976).
30. G. L. Ellman, K. D. Courtney, V. Andres, Jr. and R. M. Featherstone, *Biochem. Pharmac.* **7**, 88 (1961).
31. P. J. Garry and J. I. Routh, *Clin. Chem.* **11**, 91 (1965).

32. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
33. M. J. Greenberg, T. Roop and S. D. Painter, *Gen. Pharmac.* **11**, 65 (1980).
34. J. A. Watts and S. K. Pierce, *J. Cell Sci.* **34**, 193 (1978).
35. M. J. Greenberg, in *Comparative Physiology of the Heart* (Ed. F. V. McCann), pp. 250–65. Birkhäuser, Basel (1969).
36. R. B. Hill and J. W. Sanger, *Biol. Bull. mar. biol. Lab., Woods Hole* **147**, 369 (1974).
37. D. A. Price, *Soc. Neurosci. Abstr.* **5**, 537 (1979).
38. R. M. Krupka, *Biochim. biophys. Acta* **410**, 115 (1975).
39. P. W. Hochachka and G. N. Somero, *Strategies of Biochemical Adaption* p. 11. W. B. Saunders, Philadelphia (1973).
40. D. Feuvray and J. de Leiris, *J. molec. cell. Cardiol.* **5**, 63 (1973).
41. D. Feuvray and J. de Leiris, *Eur. J. Pharmac.* **16**, 8 (1971).
42. M. Shlafer and A. M. Karow, *Ann. N.Y. Acad. Sci.* **243**, 111 (1975).