Br. J. Pharmac. (1971), **42**, 412–422.

Effect of eserine on the chick biventer cervicis preparation

THE LATE D. W. BALDWIN* AND E. LESSER

Department of Pharmacology, Chelsea College, London SW3

Summary

1. The relationship between the capacity of the chick biventer preparation, both intact tissue and homogenate, to inactivate acetylcholine and the ability of eserine to increase the sensitivity of the tissue to acetylcholine have been investigated.

2. At concentrations of eserine of $2{\cdot}69 \times 10^{-9}$M and $2{\cdot}69 \times 10^{-8}$M the capacity of the whole tissue to inactivate acetylcholine is reduced by 5% and 15% respectively. These concentrations of eserine increase the sensitivity of the preparation to acetylcholine by factors of 2 and 4 respectively, without a change of slope in regression lines.

3. At concentrations of eserine of $2{\cdot}69 \times 10^{-7}$M and $2{\cdot}69 \times 10^{-6}$M the capacity of the whole tissue to inactivate acetylcholine is reduced by 40% and 80% respectively, and the sensitivity to acetylcholine increased by factors of 70 and 800 respectively, along with marked increases in the slopes of the regression lines.

4. An attempt has been made to quantify these differences by proposing a model in which cholinesterase in the tissue is regarded as a network, preventing the access of a large fraction of the acetylcholine to its site of action.

5. It is suggested that, whereas the increase in sensitivity to acetylcholine at eserine concentrations of $2{\cdot}69 \times 10^{-9}$M and $2{\cdot}69 \times 10^{-8}$M can be interpreted as an anticholinesterase effect (which is still present at higher concentrations), that seen at the higher concentrations may represent a direct action of eserine on the tissue.

6. It is further suggested that there are barriers to penetration in intact tissue to both substrate and inhibitor, which invalidate attempts to extrapolate results from homogenates.

Introduction

The study of cholinesterase (ChE) activity in muscle has usually been carried out, using tissue homogenates, with substrate concentrations several orders of magnitude greater than those which produce a shortening of the muscle (see, for example, review by Hobbiger, 1968, p. 304).

The isolated chick biventer cervicis nerve-muscle preparation, first described by Ginsborg & Warriner (1960), is unusual in its insensitivity to acetylcholine (ACh). Contractions of this muscle are obtained only with high concentrations of ACh. Eserine has been shown both to potentiate muscle twitch, elicited by nerve stimula-

* Died of injuries received in an aeroplane crash in Uganda in May 1971.

tion, and to increase sensitivity to ACh by as much as a 100-fold (Lesser, 1967). Similar effects are produced by neostigmine (Ginsborg & Warriner, 1960).

These observations suggested the possibility of using this tissue for determining ChE activity using substrate (ACh) concentrations normally required to evoke an effect on the isolated tissue, and hence of establishing what degree of correlation exists between the increase in sensitivity of the intact isolated preparation to ACh and inhibition of the tissue ChE activity.

Methods

Animals

Male chicks of Warren strain, weighing 60–140 g and 11–30 days old, were used throughout.

Isolated biventer cervicis nerve-muscle preparation

Chicks were anaesthetized with sodium pentobarbitone, injected into a gastrocnemius muscle. The preparation was isolated (Ginsborg & Warriner, 1960), set up in Krebs solution in a 10 ml organ bath at 37° C and gassed with a mixture of 5% CO_2 in O_2. The tendon, with nerve ensheathed, was passed through a platinum electrode assembly (Bulbring, 1946) and attached to a Starling heart lever at a tension of approximately 1·5 g and an 8-fold magnification. The preparation was stimulated at intervals of 10 s with rectangular wave shocks (duration 2–9 ms) causing maximal twitch responses.

During measurement of the effect of depolarizing drugs the twitch responses to stimulation were recorded for a control period of 2 min, followed by a drug contact period of 3 min (Fig. 1). After washing out the drug the muscle was rested, without stimulation, for 4 min when using ACh, succinylcholine (SCh) and carbachol (CCh), or for 5 min when using decamethonium (C_{10}) before the cycle was recommenced. Only one of the depolarizing drugs was used on each preparation, and the first two contractures obtained were disregarded for purposes of measurement. Two responses to each of two or three different dose levels were recorded.

Eserine was added and left in contact with the tissue for 30 min before repeating the sequence described above. The effects of two concentrations of eserine were usually determined on an individual tissue, always starting with the lower concentration. The maximal contracture of the tissue to the depolarizing drug was determined at the end of each experiment.

Preparation of the biventer cervicis muscle for manometric determination of cholinesterase activity

Muscles were removed from anaesthetized chicks as previously described and treated in one of two ways: (a) as intact tissue, muscles being placed immediately in the main compartment of the reaction flask, which already contained Krebs' solution with or without eserine; the muscles were about 0·8 mm thick; (b) as homogenate, the tissue being minced with scissors, mixed with Krebs' solution and homogenized with a teflon pestle. The homogenizer tube was surrounded with ice and the pestle was rotated at the lowest practicable speed and in short periods of not longer than 5 seconds. It was not possible to disrupt the tissue completely, thin sheets of tendon remaining intact. The contents of the tube were tipped into the main compartment of the flask, which already contained the required amount of eserine. Each flask contained all the material from a single muscle.

Any tissue remaining in the tube was washed into the flask with Krebs solution as required to make up the final fluid volume of the flask, which varied in different experiments because of the need to provide adequate substrate at the lower concentrations.

Manometric determination of cholinesterase activity

All measurements were made on a constant-pressure Gilson Differential Respirometer at 37° C, with an atmosphere of 5% CO_2 in O_2, Krebs solution being used as the bicarbonate buffer. ChE activity was expressed as (μl CO_2 at STP/100 mg wet weight of tissue)/30 min, taken from the moment when the substrate solution was tipped from the side-arm into the main compartment of the flask. In experiments with homogenized tissue at the lowest substrate concentrations it was necessary to take readings at intervals of 2·5 min and to obtain a 30 min value by extrapolation. In all experiments four flasks, without tissue but containing Krebs solution and ACh with or without eserine, were included as controls.

Determination of ChE activity by bioassay

The gas yields at low substrate concentrations were too low for manometric measurement, and the ChE activity of intact muscles was, therefore, estimated by bioassay of residual ACh on guinea-pig ileum in Krebs solution containing neostigmine (5 μg/l.) and morphine (10 mg/l.) at 32° C (Paton, 1957). Standard ACh solutions contained the same concentrations of eserine as the test solutions. Incubations were carried out under the same conditions as in manometric experiments, but at the end of the reaction time the flasks were immediately detached from the apparatus, the tissue removed from the flask, which was cooled, and the ACh content of the medium assayed within 2 hours. No ChE activity was detectable at substrate concentrations below $7{\cdot}33 \times 10^{-6}$M ACh.

Isolated biventer cervicis preparations, pretreated with $2{\cdot}69 \times 10^{-6}$M eserine, were also used for the bioassay of ACh remaining in some flasks after reaction for 30 min in the differential respirometer. Fair agreement was obtained between the estimates of ACh hydrolysis obtained by the manometric method and by the bioassay, even at the lower limit of substrate concentration in the manometric apparatus, that is, $7{\cdot}33 \times 10^{-5}$M ACh, indicating that the gas evolved and measured manometrically was the product only of ACh hydrolysis. A *t* test showed no significant difference ($P=0{\cdot}05$) between the amounts of ACh hydrolysed as calculated by the two methods.

Drugs and solutions

The Krebs solution had the following composition: (mM) NaCl 120; KCl 4·7; $NaHCO_3$ 25; glucose 11; KH_2PO_4 1·2; $MgSO_4$ 1·2; $CaCl_2$ 1·9; pH 7·3; in deionized water, saturated with 5% CO_2 in O_2 before use. All concentrations of drugs are expressed as w/v of the salt, or as molar concentrations. Stock solutions were stored at 4° C in the dark. All drug solutions were made in 0·9% w/v NaCl in deionized water. The drugs used were: acetylcholine iodide (ACh), B.D.H.; succinylcholine chloride (SCh), Scoline, Allen & Hanburys; carbamylcholine chloride (CCh) and decamethonium iodide (C_{10}), Koch-Light; physostigmine sulphate (Eserine, Es), Burroughs Wellcome; pentobarbitone sodium, Nembutal, Abbot; neostigmine methylsulphate, Prostigmin, Roche; morphine hydrochloride, May & Baker.

Results

Effect of eserine on the responses of the isolated biventer cervicis to depolarizing drugs

With each of the depolarizing drugs (ACh, CCh, SCh or C_{10}) the twitches were well maintained in the presence or absence of eserine, except where the contractures were near maximal. With $2{\cdot}69 \times 10^{-7}$M and $2{\cdot}69 \times 10^{-6}$M eserine, but not with lower concentrations, approximately 2-fold increases in maximal twitch height were observed (Fig. 1); $2{\cdot}69 \times 10^{-5}$M eserine, however, completely abolished the increase in the height of the twitches due to the previous exposure to $2{\cdot}69 \times 10^{-6}$M eserine. The 'fade' in contracture usually observed with ACh was not affected by $2{\cdot}69 \times 10^{-9}$M eserine, was usually decreased by $2{\cdot}69 \times 10^{-8}$M eserine and was prevented by higher concentrations of eserine (Fig. 1). No 'fade' was observed with the other depolarizing drugs.

The effects of eserine on the responses to depolarizing drugs were expressed quantitatively from the changes in the lines relating percentage of maximal contracture to log molar concentration. Estimates, usually by interpolation, of drug concentrations causing 20, 50 and 80% of maximal contracture are shown in Fig. 2, while in Table 1 the results are expressed as R, the ratio drug concentration in the absence of eserine to drug concentration in the presence of eserine. Increased sensitivity to ACh in the presence of $2{\cdot}69 \times 10^{-9}$M and $2{\cdot}69 \times 10^{-8}$M eserine was not accompanied by a change in slope of the regression lines; with $2{\cdot}69 \times 10^{-7}$M and $2{\cdot}69 \times 10^{-6}$M eserine, however, increases in sensitivity were now associated with increases in slope. It is noteworthy that, compared with $2{\cdot}69 \times 10^{-6}$M eserine, $2{\cdot}69 \times 10^{-5}$M caused a fall in both R and slope values. In the absence of eserine the slopes of the regression lines of CCh, C_{10} and SCh were greater than those for ACh, but in the presence of $2{\cdot}69 \times 10^{-6}$M eserine a decrease in slope occurred, accompanied by significant but relatively small increases in sensitivity.

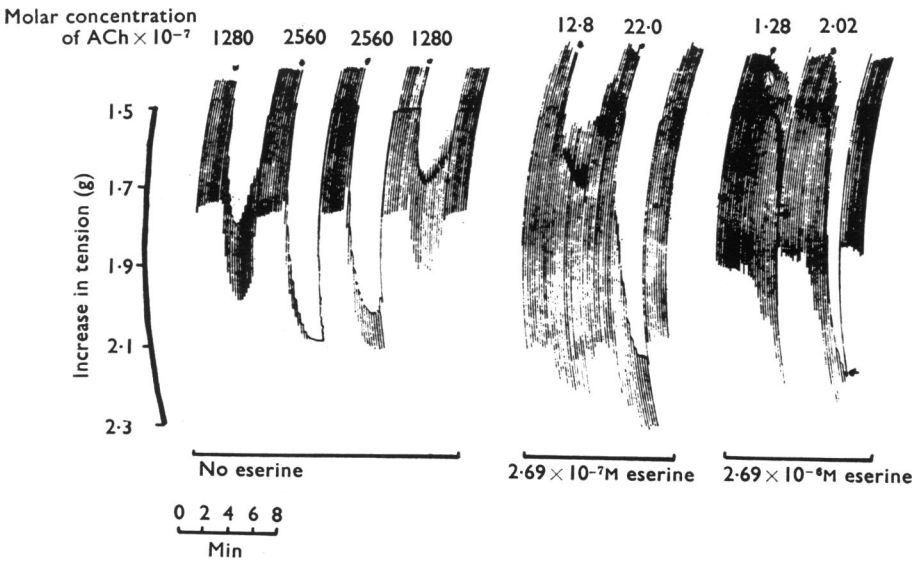

FIG. 1. Responses of the isolated chick biventer preparation to acetylcholine in the absence and presence of eserine.

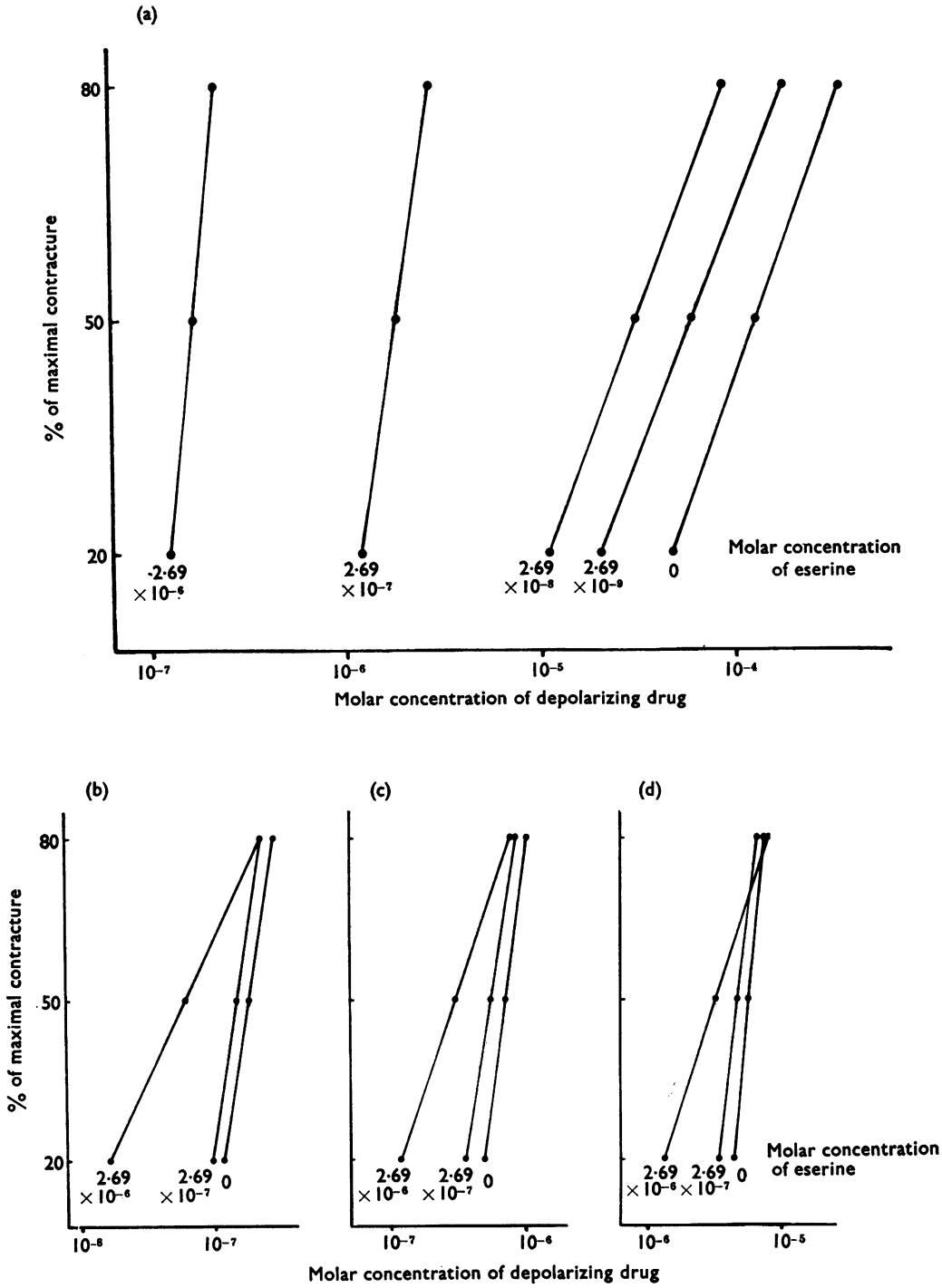

FIG. 2. Regression lines for (a) acetylcholine, (b) decamethonium, (c) succinylcholine, and (d) carbachol in the absence and presence of eserine. Each point is the mean of four or more observations.

TABLE 1. *Effect of eserine on the dose-response curves of depolarizing drugs*

	Before eserine		Molar concentrations of eserine							
			$2{\cdot}69\times10^{-9}$		$2{\cdot}69\times10^{-8}$		$2{\cdot}69\times10^{-7}$		$2{\cdot}69\times10^{-6}$	
Drug	Molar conc. for 50% maximal contracture ± S.E. (*n*)	*b* ± S.E. (*n*)	*R* ± S.E. (*n*)	*b* ± S.E. (*n*)	*R* ± S.E. (*n*)	*b* ± S.E. (*n*)	*R* ± S.E. (*n*)	*b* ± S.E. (*n*)	*R* ± S.E. (*n*)	*b* ± S.E. (*n*)
ACh	$1{\cdot}3\pm0{\cdot}2\times10^{-4}$ (10)	70±6 (10)	2·1±0·5 (4)	64±6 (4)	4·2±1·2 (4)	67±7 (4)	71±8 (4)	107±15 (4)	780±150 (4)	260±50 (4)
CCh	$5{\cdot}6\pm0{\cdot}9\times10^{-6}$ (10)	270±25 (10)					1·2±0·1 (5)	210±11 (5)	1·8±0·1 (6)	80±17 (6)
SCh	$7{\cdot}1\pm0{\cdot}4\times10^{-7}$ (4)	190±15 (4)					1·3±0·01 (4)	160±13 (4)	2·3±0·3 (4)	74±5 (4)
C_{10}	$1{\cdot}8\pm0{\cdot}3\times10^{-7}$ (7)	160±29 (7)					1·2±0·1 (4)	170±22 (4)	3·0±0·4 (4)	54±3 (4)

R=The ratio of concentration of depolarizing drug in the absence, compared with that in the presence of eserine at 50% of maximal contracture. *b*=The slope of the straight line regression of the increase in response, as a percentage of maximal contracture, against log/concentration of drug. S.E.=Standard error of the mean, *n*=number of determinations for each mean.

Two determinations of the effect of $2{\cdot}69\times10^{-5}$M of eserine on the response to ACh, gave a value for *R* of about 200, the slope of the regression lines being about 160.

Effect of eserine on the cholinesterase activity of variously prepared tissue

Considerable differences were observed between the ChE activities of homogenized and intact tissue and in their inhibition by eserine (Fig. 3), differences that are most simply explained in terms of the accessibility of substrate and inhibitor to the enzyme. For example, the optimal substrate concentration for the homogenized tissue was $7{\cdot}33 \times 10^{-3}$M ACh, whereas the optimal concentration for the intact tissue was at least 10 times as great. Furthermore, while inhibition of ChE activity was

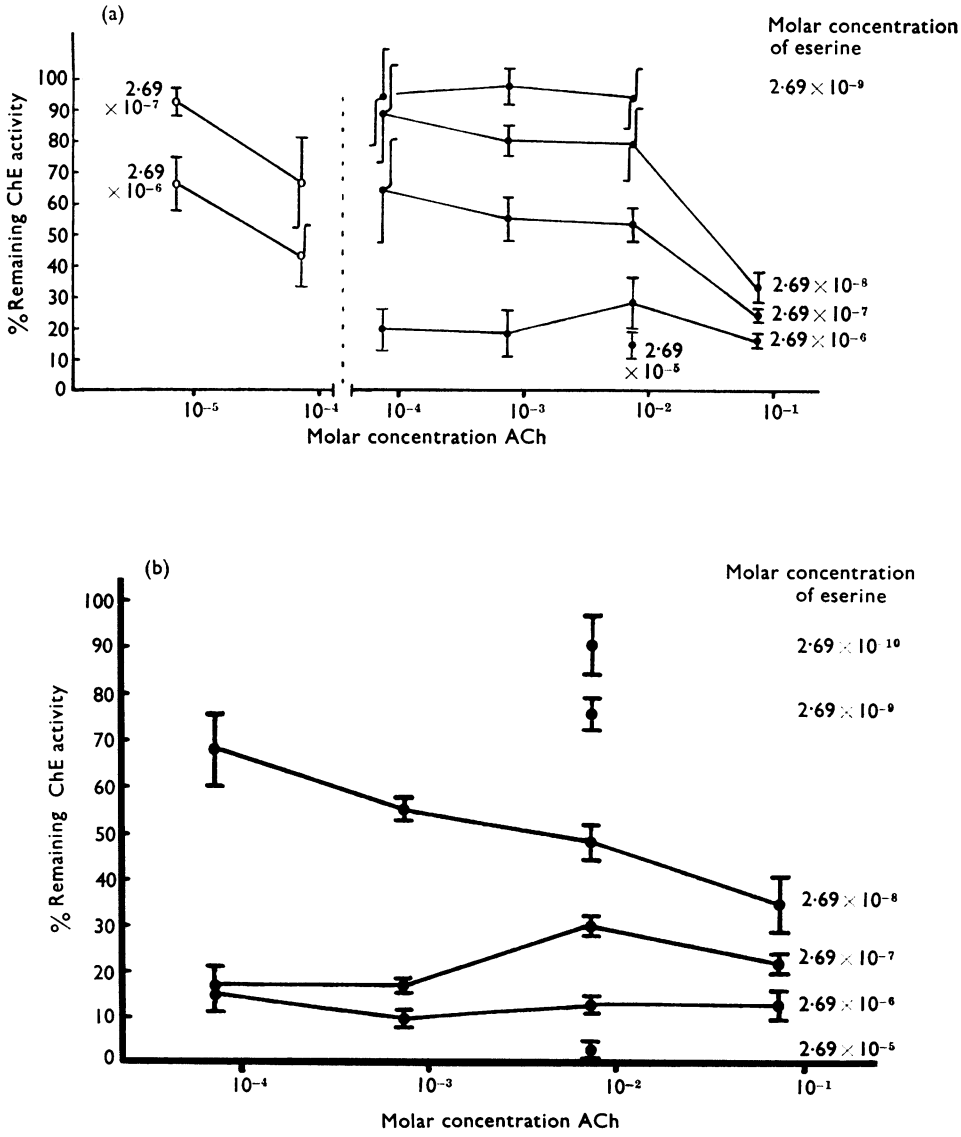

FIG. 3. Estimations of % ChE activity remaining in the presence of eserine, using manometric and bioassay techniques, in (a) intact tissue: (—○—) are means, from three or more determinations, obtained by bioassay of ACh on guinea-pig ileum ; (—●—) are means from not less than six determinations by the manometric method ; (b) homogenized tissue: manometric method only. Standard errors are indicated by vertical bars.

observed with high concentrations of substrate in homogenized tissue, this was not seen in intact tissue (Fig. 4). Again, in order to produce the same percentage of inhibition, intact tissue required an eserine concentration approximately 10 times as great as that in homogenates (Fig. 3). The pI_{50} values for eserine inhibition of ChE activity in the presence of $7{\cdot}33 \times 10^{-3}$M ACh were found to be 7·7 and 6·4, and in the presence of $7{\cdot}33 \times 10^{-5}$M ACh, 7·1 and 6·1 for homogenized and intact tissue, respectively. It appears therefore that increased disruption of the tissue was associated with greater ChE activity and also with greater inhibition of that activity by eserine.

Finally, it can be seen that the degree of inhibition of ChE activity by eserine decreases with decreasing substrate (ACh) concentration (Fig. 3), a feature characteristic of coupling inhibition (Webb, 1963).

Discussion

In determining the ChE activity of the chick biventer muscle every effort was made to duplicate in the reaction flask the conditions of the isolated preparation in the organ bath, by using the same fluid medium, temperature and gas mixture in both situations. Both intact tissues and homogenates were used as enzyme source.

More important than the considerable difference observed between the ChE activities of intact and homogenized tissues was the difference in the degree of inhibition by eserine, which is probably due to barriers to the drugs in the intact tissue. Assuming that ACh had easy access to ChE in the homogenates, then a comparison of the ACh concentrations yielding similar gas outputs in intact and homogenized tissue could be made. This would seem to indicate that the concentrations of ACh in the intact tissue, derived from the added drug, attained at the ChE sites was about one-hundredth that in the surrounding fluid (Fig. 4). By the same argument a comparison of pI_{50} values at $7{\cdot}33 \times 10^{-5}$M ACh suggests that the concentration of eserine within the intact tissue reached only one-tenth that in the surrounding fluid.

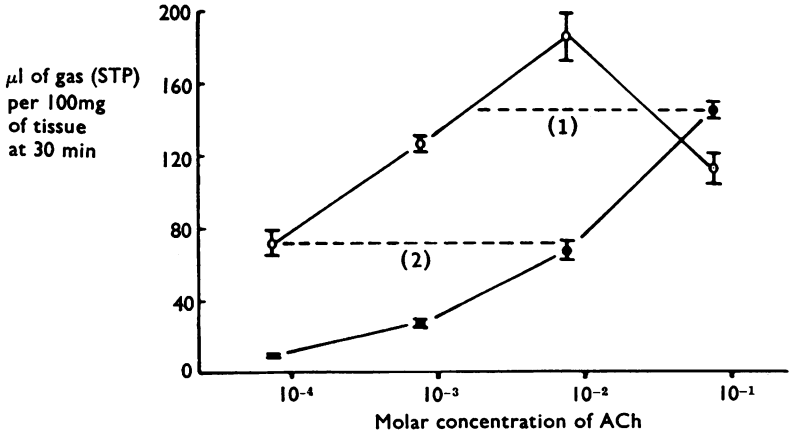

FIG. 4. Comparison of substrate concentrations required to yield the same gas output (cholinesterase activity) in intact (—●—) and homogenized (—○—) preparations of chick biventer muscle. Each point represents the mean of not less than six determinations, and the standard errors are indicated by the vertical bars. Note: the broken lines show that the concentrations of ACh in the bulk phase for equal gas output are (1) 49, and (2) 110 times as great with intact tissue as with homogenized tissue.

It may, however, be more valid to compare pI_{50} values at ACh concentrations that gave equal hydrolysis rates for both intact tissue and homogenates, in which case the concentration of eserine within the tissue would appear to reach one-fifth that in the surrounding fluid. These findings emphasize the inherent difficulty of attempts to correlate the effects of anti-cholinesterase drugs on intact tissues with their inhibition of the ChE activity of the homogenized tissue or purified enzyme preparation.

Turning now to the intact preparation, it was calculated from manometric experiments that, even with the largest tissue used in the organ bath, less than 5% of the ACh required in the bulk phase to produce a 50% maximal contracture in the absence of eserine could be hydrolysed during the contact time of 3 minutes. The pronounced effect of eserine in increasing the sensitivity of the isolated preparation to ACh is unlikely therefore to be due to its preventing the slight decrease of the ACh concentration in the bathing fluid that would otherwise occur.

In considering an alternative interpretation of the results, a model is proposed to describe the events within the tissue, according to which ACh is prevented from reaching its site(s) of action by a network of ChE. The assumption is made that 50% maximal contracture in both the presence and absence of eserine requires the same amount of ACh to be present at its site(s) of action. Let C=the total amount of ACh available to produce 50% maximal contracture in the absence of eserine; R=the ratio of the concentration of ACh in the absence compared with that in the presence of eserine at 50% maximal contracture (Table 1); u=the uninhibited fraction of ChE in the presence of $1{\cdot}3\times10^{-4}$M ACh; and x=the apparent fraction of ACh prevented by the ChE network from reaching the site(s) of action. Our assumption can then be expressed in the form $C(1\text{-}x)=\frac{C}{R}(1\text{-}ux)$ . . . (1), in which the left hand side of the equation represents the situation in the absence of eserine and the right hand side that in its presence. Re-arranging (1), $x=\frac{R\text{-}1}{R\text{-}u}$, and thus the values of x from all four concentrations of eserine can be calculated (Table 2).

TABLE 2. *Effect of eserine on the functioning of the isolated chick biventer cervicis muscle*

Molar concentration of eserine	u	R	x (calculated)	$\frac{1}{(1-x)}$
$2{\cdot}69\times10^{-9}$	0·95	2	0·952	21
$2{\cdot}69\times10^{-8}$	0·85	4	0·952	21
$2{\cdot}69\times10^{-7}$	0·60	70	0·994	167
$2{\cdot}69\times10^{-6}$	0·20	800	0·999	1,000

See text for explanation

TABLE 3. *Increases in sensitivity to ACh of the isolated chick biventer cervicis preparation due to eserine*

Molar concentration of eserine	$2{\cdot}69\times10^{-9}$	$2{\cdot}69\times10^{-8}$	$2{\cdot}69\times10^{-7}$	$2{\cdot}69\times10^{-6}$
Total increase in sensitivity to ACh (R)	2	4	70	800
Predicted increase in sensitivity to ACh due to inhibition of cholinesterase (R')	2	4	9	17
Additional increase in sensitivity to ACh (R/R')	1	1	8	47

See text for explanation

It should be noted that the equation does not take account of such factors as the influence of substrate (ACh) and enzyme-inhibitor concentrations upon the pattern and volume of distribution of ACh in the tissue, and it is presented as a first approximation. If the hypothesis is correct the results obtained with $2{\cdot}69 \times 10^{-9}$M eserine (in the presence of which ChE activity is reduced by 5% while the concentration of ACh required to elicit 50% maximal contracture is halved) suggest that only one out of every twenty-one molecules of ACh reaching the ChE network penetrates to the site(s) of action in the absence of eserine ; the results obtained with $2{\cdot}69 \times 10^{-8}$M eserine lead to the same conclusion (Table 2). (It is perhaps of interest to note that in the absence of eserine the isolated tissue is 23 times as sensitive to CCh as to ACh.) By the same reasoning the results obtained with $2{\cdot}69 \times 10^{-7}$M eserine suggest that only one out of every 167, and with $2{\cdot}69 \times 10^{-6}$M eserine only one out of every 1,000 molecules of ACh reaching the ChE network in the absence of eserine penetrates to the site(s) of action. Given the original assumption, and if the hypothesis is correct, the apparent fraction of molecules of ACh penetrating the ChE network in the absence of eserine, as calculated from the results obtained in the presence of all four concentrations of eserine, should be the same. As this is not the case it must be inferred that the hypothesis is incorrect.

The ratios obtained with $2{\cdot}69 \times 10^{-9}$M and $2{\cdot}69 \times 10^{-8}$M eserine are the same, however. It may be therefore that the approach used does hold at these lower concentrations of eserine ; other observations lend support to this interpretation. Thus, the effect of eserine on the dose-response curves of ACh (Fig. 1a and Table 1) is to cause shifts in the regression lines, due to $2{\cdot}69 \times 10^{-9}$M and then $2{\cdot}69 \times 10^{-8}$M eserine, entailing no change of slope. The larger shifts due to $2{\cdot}69 \times 10^{-7}$M and $2{\cdot}69 \times 10^{-6}$M, on the other hand, are accompanied by a 2·5-fold and 4-fold increase in slope, respectively, of the regression lines.

There is, therefore, a change in the pattern of eserine action at higher concentrations, leading both to a much higher sensitization of the tissue to ACh and to a steepening of its dose-response curve. Table 3 expresses the former change, showing the total increase of sensitivity, the increase explicable on the simple model described, and the further factor of increase which appears at higher concentrations. Two types of explanation may be advanced for this. The first is that eserine has an additional action, increasing the sensitivity of the tissue to ACh by some means other than by cholinesterase inhibition. Such a mechanism would have to be selective to ACh, since any sensitization to decamethonium, succinylcholine and carbachol is relatively small. The second is that the model used is too simple. It assumes, in particular, that the contractural effect of a given quantity of acetylcholine reaching the receptors is the same, regardless of the distribution of that quantity throughout the tissue. In fact, from known diffusion coefficients and the dimensions of the muscles used, it must be supposed that in the absence of eserine the contraction is chiefly due to the outer fibres responding to relatively high concentrations of ACh, the inner fibres behaving as a passive splint ; but in the presence of sufficient eserine, the deeper fibres would be progressively recruited, and lower concentrations of ACh would be effective. Some support for this is found in the steepening of the dose-response curve, since, if the ACh concentration became more even throughout the muscle during an exposure, the fibres would respond more uniformly. But the position is very complex, with each plane of fibres liable to be at a different point on the ACh dose-response curve ; it would require analysis on a finer scale to clarify the position.

We are grateful to Professor M. Ginsburg for his many suggestions for improving this paper and to Miss S. Lawrence for technical assistance in its preparation.

REFERENCES

BULBRING, E. (1946). Observations on the isolated phrenic nerve-diaphragm preparation of the rat. *Br. J. Pharmac. Chemother.*, **1**, 38–61.

GINSBORG, B. L. & WARRINER, J. (1960). The isolated chick biventer cervicis nerve-muscle preparation. *Br. J. Pharmac. Chemother.*, **15**, 410–411.

HOBBIGER, F. (1968). In: *Recent Advances in Pharmacology*, pp. 281–310. London: J. and A. Churchill.

LESSER, E. (1967). A study of peripheral receptor systems, which react to acetylcholine, in the cat, the fowl, the guinea-pig, the rat and the frog, Ph.D. thesis, University of London.

PATON, W. D. M. (1957). The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **12**, 119–127.

WEBB, J. L. (1963). Variation of fractional inhibition with substrate concentration for different types of inhibition. In: *Enzyme and Metabolic Inhibitors*, vol. **1**, p. 63, fig. 3–5. New York: Academic Press.

(*Received January* 23, 1970)