Brit. J. Pharmacol. (1962), **18**, 550-562.

ACTIONS OF SOME ANTICHOLINESTERASES ON THE SMOOTH MUSCLE OF THE CHICK AMNION

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(*Received January* 27, 1962)

The actions of both reversible and irreversible anticholinesterase drugs on the nerve-free smooth muscle of the chick amnion are described. The tertiary compound physostigmine and the irreversible inhibitor di*iso*propylfluorophosphonate were found to be active in causing contractions, whereas the quaternary compound neostigmine and the irreversible inhibitor Mipafox were only slightly active in this respect. Manometric studies showed that all four compounds were highly active, however, in inhibiting the cholinesterase found in the chick amnion. The effects produced by these compounds in the organ bath are thought to be due to the accumulation of endogenously produced acetylcholine within the tissue. Evidence for an intracellular action of endogenously produced acetylcholine in the smooth muscle of the chick amnion is discussed.

Acetylcholine is endogenously produced unassociated with nervous elements in a number of spontaneously active tissues. This has been shown for rabbit auricles (Comline, 1946; Bülbring & Burn, 1949), the gill plates of mytilus (Bülbring, Burn & Shelley, 1953) and rabbit intestine (Feldberg & Lin, 1950). The presence of an acetylcholine-like substance in extracts of the nerve-free smooth muscle of the chick amnion has previously been demonstrated (Cuthbert, 1960). Anticholinesterases are thought to act in spontaneously active tissues by allowing accumulation of endogenously produced acetylcholine to occur (see reviews by Burn, 1954, and Feldberg, 1950).

Suggestions that acetylcholine may act at sites other than the cell membrane have appeared in the literature. Bülbring & Burn (1949) have suggested an action on the choline acetylase system in rabbit auricles. Hill (1927) described fine nerve fibres which penetrated the smooth muscle cells of guinea-pig ileum suggesting that transmission may be an intracellular process at this site. Acetylcholine shows a slight effect on electrically stimulated glycerinated muscle fibre models in the presence of adenosine triphosphate (Fujino, Matsushima, Muroya, Yabu, Yamaguchi & Takahashi, 1960). The aim of this present work was to obtain information on the action of anticholinesterase drugs on the smooth muscle of the chick amnion in the hope of clarifying the site of action of the endogenously produced acetylcholine.

METHODS

The experiments were performed on amniotic membranes dissected from hens' eggs incubated at 37° C for periods varying from 10 to 17 days. The eggs were not turned during incubation.

Isolated chick amniotic membranes. The amniotic membranes were suspended in a 12 ml. organ bath at 37° C and isotonic contractions of the musculature were recorded on a smoked drum or with an ink writing lever. A light straw lever was used with a magnification of approximately 100:1. The resting tension was altered according to the age of the amniotic membranes; with membranes of 10 or 11 days the lever was balanced, whereas, for the older membranes, about 50 mg tension was applied. Hanks' balanced salt solution, gassed with air, was used throughout. This solution had the following composition (g/l.): sodium chloride, 8.00; potassium chloride, 0.40; calcium chloride, 0.14; magnesium sulphate heptahydrate, 0.10; magnesium chloride hexahydrate, 0.10; disodium hydrogen phosphate dihydrate, 0.06; potassium dihydrogen phosphate, 0.06; glucose, 1.00; and sodium bicarbonate 0.35.

Manometric determinations of cholinesterase. The cholinesterase activity of amniotic membranes was estimated manometrically at 37° C in an atmosphere of 95% nitrogen and 5% carbon dioxide. The enzyme preparation was prepared by grinding the tissue in an all-glass homogenizer in distilled water. The enzyme preparation and inhibitor were placed in the main compartment of the flask and the substrate, dissolved in 0.5 ml. of distilled water, placed in the side arm. The amount of tissue used in each flask was variable, but did not exceed 300 mg wet weight. When α-naphthyl acetate was used as a substrate for cholinesterase, an amount sufficient, if soluble, to produce a concentration of 0.03 M was weighed into the flasks, and the enzyme was placed in the side arm. Inhibition with irreversible anticholinesterases was obtained by incubation of the enzyme preparation for 1 hr at 37° C before placing in the main compartment of the flask. The cholinesterase activity of hen plasma was performed on a sample of plasma diluted 5 times with distilled water; 0.5 ml. of this dilution was used in each flask. The estimations were carried out in bicarbonate buffer previously adjusted to *p*H 7.6, the final concentration of sodium bicarbonate being 0.033 M.

Staining for cholinesterase in hen plasma and chick amnion. Cholinesterase was stained by a modification of the method of Lewis (1958) in which α-naphthyl acetate is used as the substrate for cholinesterase. A working buffer solution (*p*H 8.1) was prepared by mixing 0.2 M tris buffer 10 ml., N hydrochloric acid 1 ml. and distilled water 9 ml.

The incubation solution contained working buffer solution 3 ml., distilled water 17 ml., brentamine fast red T.R. salt 25 mg., α-naphthyl acetate 10 mg.

The ingredients of the incubation solution were shaken vigorously for 30 sec, filtered and used immediately for staining hen plasma and amniotic membranes, as described in Results.

Compounds used. Acetylcholine chloride; atropine sulphate; physostigmine salicylate; neostigmine methylsulphate; di*iso*propylfluorophosphonate (dyflos); bismono*iso*propylaminofluorophosphine oxide (Mipafox); choline chloride; α-naphthyl acetate; brentamine fast red T.R. salt; tris (hydroxymethyl) aminomethane; disodium edetate; and ambenonium chloride. Doses of drugs are expressed as the bases unless otherwise stated.

RESULTS

The effects of drugs on amniotic smooth muscle in the isolated organ bath. The effect of acetylcholine on a 13-day preparation is shown in Fig. 1. The preparation showed moderate sensitivity to acetylcholine and the response was completely blocked by atropine at one-tenth of the acetylcholine concentration. A tenfold increase in acetylcholine concentration overcame the block. Prolonged washing over 30 min restored the response to the former level. In this preparation spontaneous contractions were reduced by atropine.

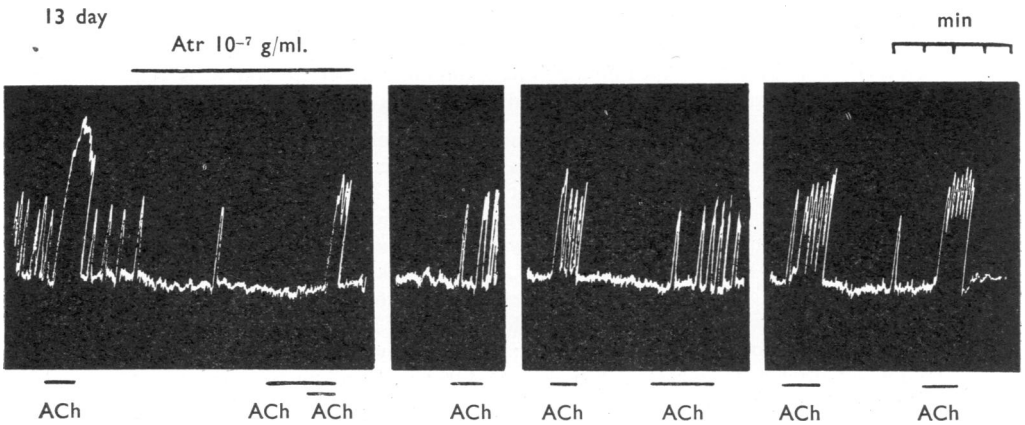

Fig. 1. Chick amnion, 13 day. Single bars below the trace indicate the contact time for 1 μg/ml., the double bars for 10 μg/ml. of acetylcholine. The presence of atropine 0.1 μg/ml. is shown by the single bar above the trace.

The variations in effects produced by physostigmine are shown in Fig. 2. The small rapid contractions seen in the 10-day (Fig. 2a) preparation were barely influenced, only a small increase in rate being produced. At eleven days (Fig. 2b) considerable increases, both in rate and tone, were produced, whereas at 13 days (Fig. 2c) the effect was exerted mainly on the tone of the preparation.

Fig. 3 shows the effects of physostigmine on seven 17-day preparations all from the same batch of eggs. Of these preparations only one showed spontaneous rhythm (Fig. 3d). Physostigmine increased the tone and exaggerated the spontaneous contractions, although there was little alteration in frequency. The preparations illustrated in Fig. 3a, b and c showed no response to physostigmine although preparation 3a responded poorly to acetylcholine in a large dose. In Fig. 3e no response to physostigmine was seen over a period of 3 min. Atropine was then added and small contractions appeared in the continued presence of the physostigmine. The contractions were improved by the addition of choline to the bath. After washing out, a response to physostigmine was seen within 3 min. Atropine was added to prevent any muscarinic effects of choline. In the experiment shown in Fig. 3f atropine 10^{-5} g/ml. did not produce contractions; concentrations of this order have been found to stimulate the amnion. In the experiment illustrated in Fig. 3g no initial response was obtained to physostigmine, and the membrane responded poorly to acetylcholine. However, after two additions of acetylcholine a response to physostigmine was apparent. Choline, in the presence of atropine, then induced an intermittent rhythm.

Occasionally a large amount of acetylcholine would induce rapid spontaneous activity in a previously inactive preparation, but only after removal of the drug. In one experiment an inactive preparation was treated with 10 μg/ml. of acetylcholine. After washing, a rapid spontaneous rhythm together with an increased tone ensued. This increased activity which persisted after further washing was uninfluenced by physostigmine, therefore resembling the effect seen in Fig. 2a. After a period of 30 min, by which time spontaneous contractions had disappeared,

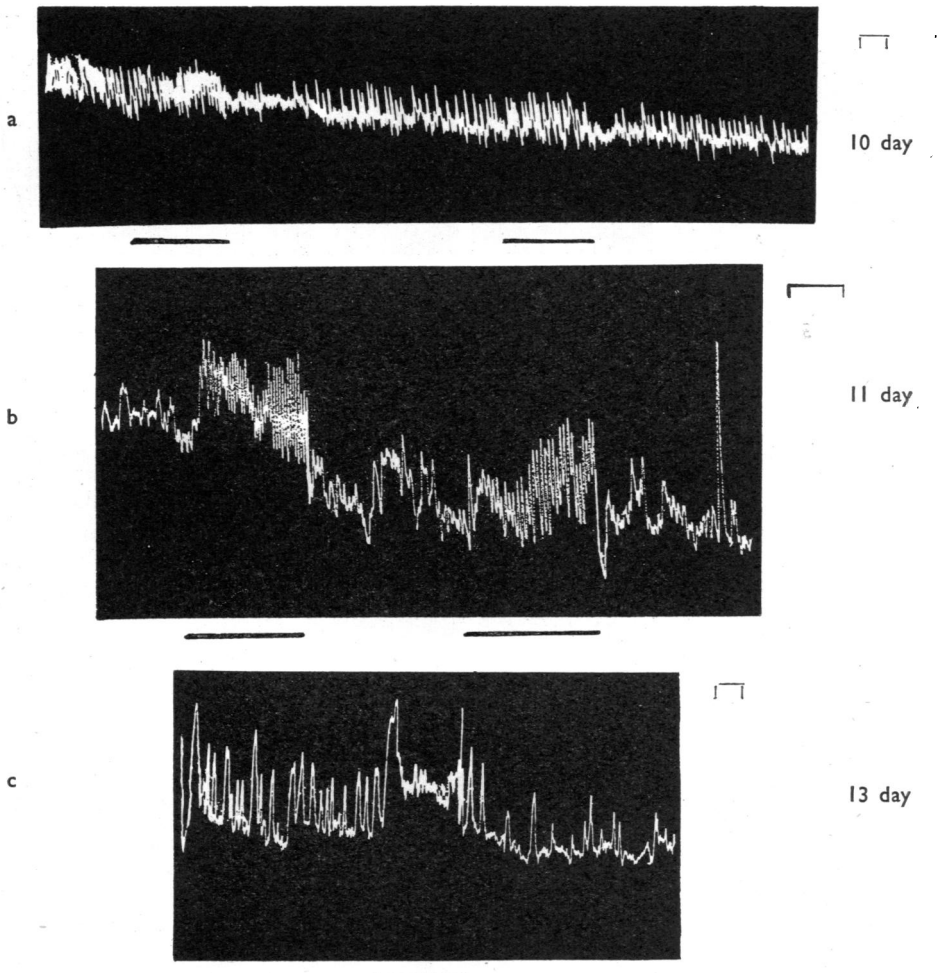

Fig. 2. Chick amnion. Doses of physostigmine used were 3.3 μg/ml. (a) and 5 μg/ml. (b and c). Contact time for the drug is shown by the bars beneath each trace, and a time mark for 1 min is shown at the upper right-hand corner of each panel.

this experiment was repeated, when an effect resembling that in the first part of Fig. 3g was seen, but the induced rhythm to acetylcholine was not marked and was increased by physostigmine.

The failure of atropine to influence the response of chick amnion to physostigmine is illustrated in Fig. 4. Standard responses to physostigmine and acetylcholine are shown, after which atropine (5×10^{-6} g/ml.) was placed in the bath for 5 min. The response to physostigmine was virtually unaffected, whereas the response to acetylcholine was abolished, returning in part only after prolonged washing. Physostigmine and neostigmine, given in concentrations of 3.3 μg/ml. for 3 min, showed marked differences in effect. In an experiment on a 14-day amnion (Fig. 5) physostigmine produced an immediate contraction whereas neostigmine caused no effect. In other experiments neostigmine was inactive when left in the organ bath for periods up

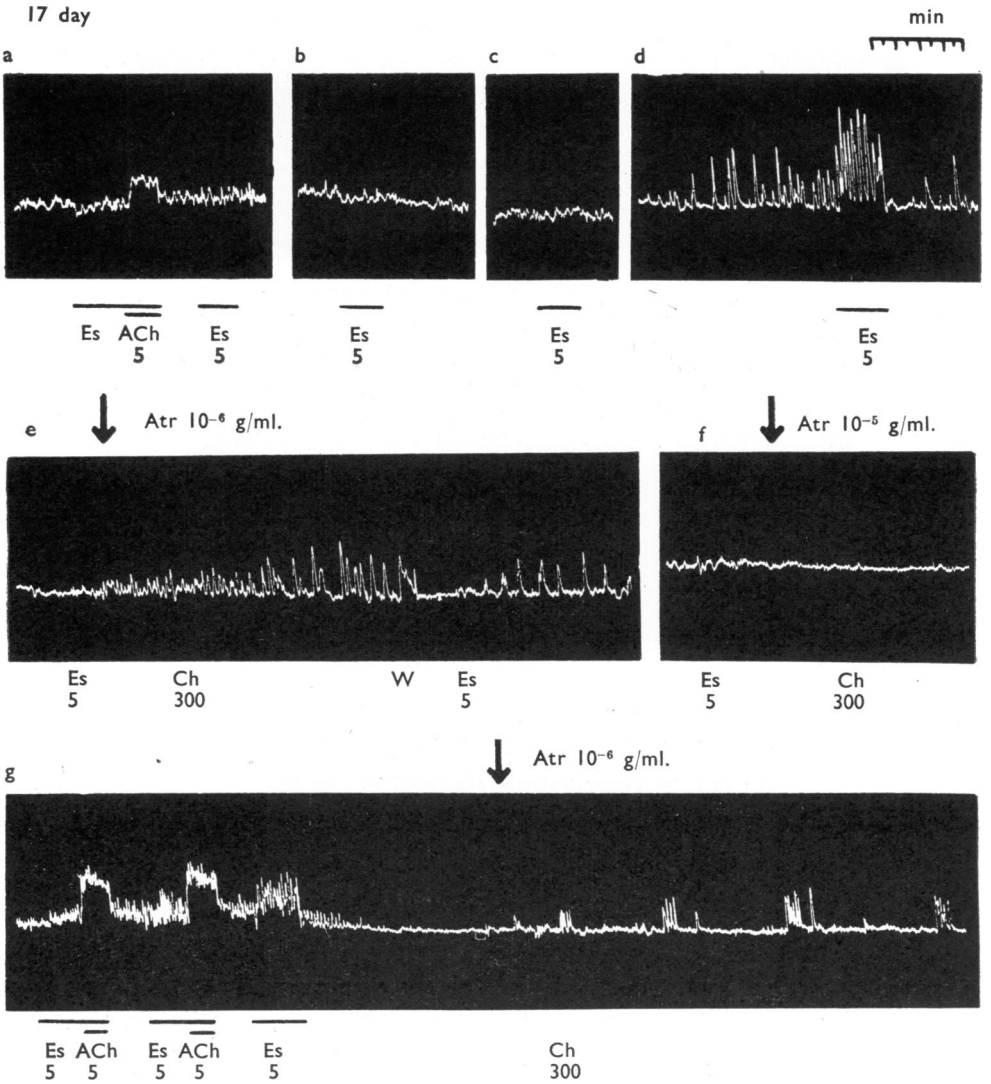

Fig. 3. Chick amniotic membranes, 17 day, showing the effects of physostigmine. Doses are expressed in μg/ml. Bars below the trace indicate the drug contact time, and where no bars are shown the drug was left in the bath. W indicates wash.

to 10 min, but occasionally a slight increase in the height and frequency of spontaneous contractions was seen. Ambenonium chloride also had no effect, and it is of interest that like neostigmine it is a quaternary compound, in contrast to physostigmine which is a tertiary amine.

Two irreversible anticholinesterase inhibitors, dyflos and Mipafox, were also tested for activity on amnion muscle. With dyflos considerable variation in response was observed over a wide range of concentrations. In some experiments no immediate response was seen, but after 5 to 20 min there was a sudden increase

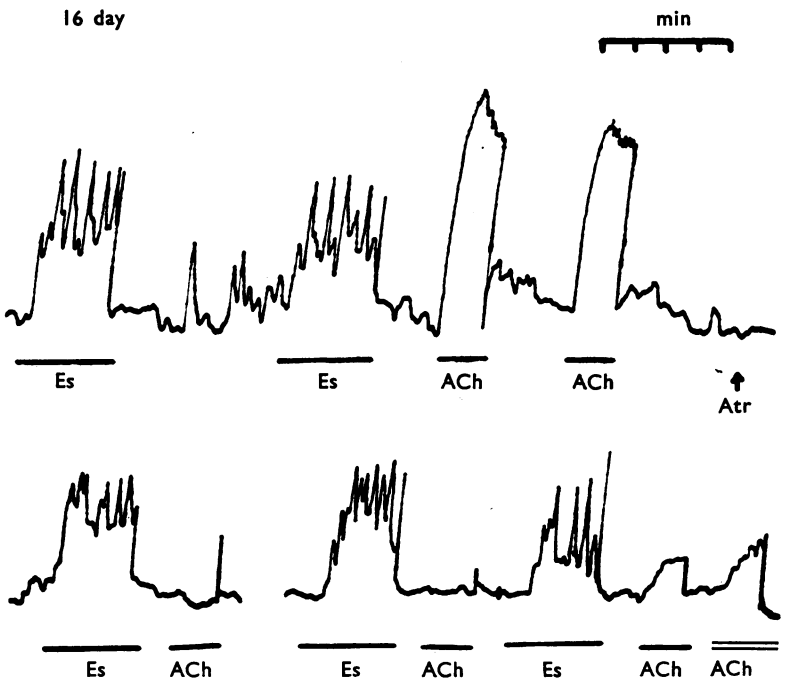

Fig. 4. Chick amnion, 16 day. Doses in μg/ml., physostigmine (Es) 5 μg/ml., acetylcholine (ACh) 2.5 μg/ml. (single bars) and 5 μg/ml. (double bars). At the arrow atropine 5 μg/ml. was placed in the bath for 5 min. Drug contact time is indicated by the length of the bars.

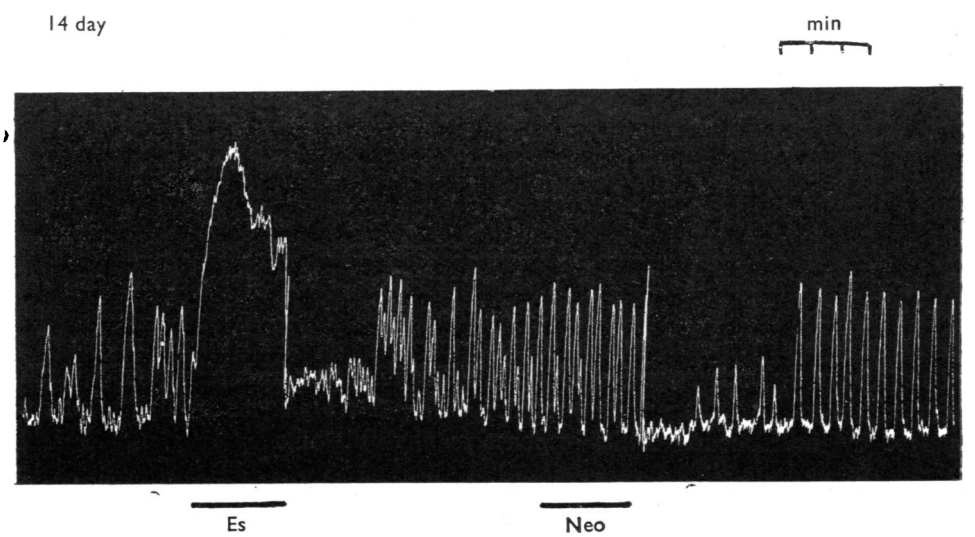

Fig. 5. Chick amnion, 14 day. Doses of physostigmine (Es) and neostigmine (Neo) were 3.3 μg/ml. given for 3 min.

in the rate of spontaneous contractions (Fig. 6a) which was maintained for some 5 to 10 min, after which activity subsided. At this time preparations still responded to physostigmine, showing there was incomplete phosphorylation of the enzyme. This type of response was produced with concentrations of dyflos as low as 1.6 μg/

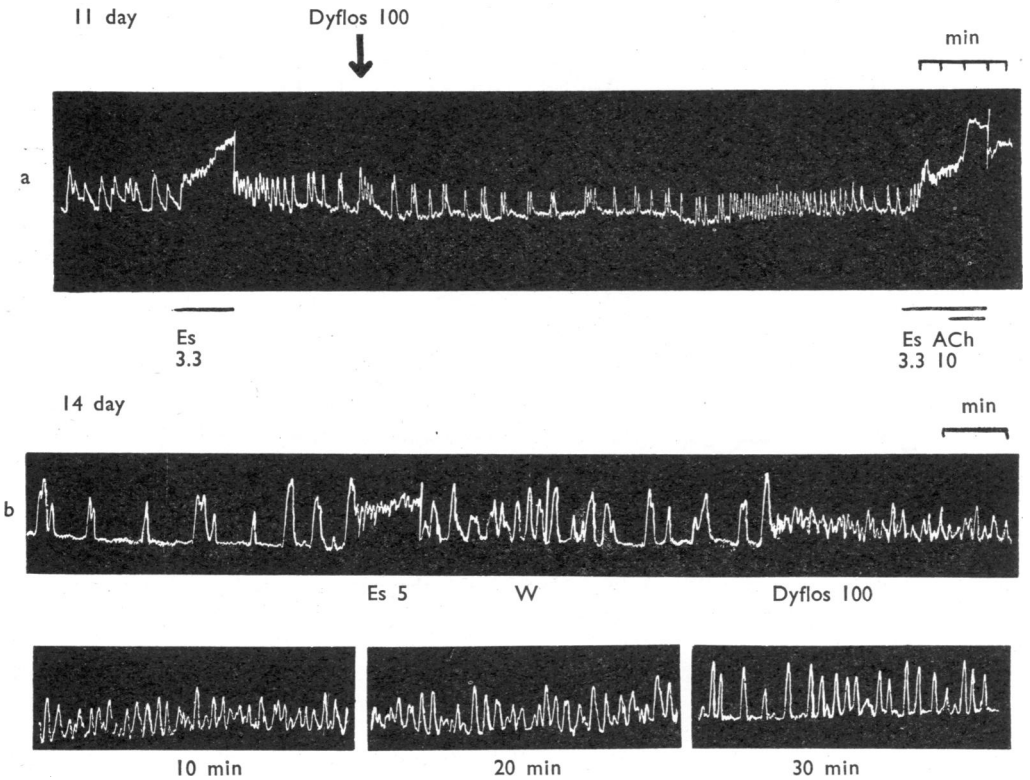

Fig. 6. Chick amnion. Records of the effects of dyflos. Doses in μg/ml. (a) 11-day amnion. Increased rhythm due to dyflos appearing after 13 min and maintained for about 5 min. (b) 14-day amnion. Dyflos produced an immediate increase in activity maintained over 30 min.

ml. and up to 100 μg/ml. With smaller doses a longer time was usually required for the appearance of rapid activity. A second type of response was an immediate prolonged increase in frequency of contractions (Fig. 6b). A third and more common type of response was the appearance of an increased muscular tone after about 1 hr of dyflos treatment at 37° C. At this time the response to physostigmine was depressed. The degree of phosphorylation of the enzyme by dyflos may be gauged by the reduction in the response to physostigmine.

The time taken for phosphorylation depends on the experimental procedure. In a series of experiments in which Mipafox at a concentration of 2.7×10^{-3} M was placed in the organ bath for 1 hr at 37° C, the response to physostigmine was considerably reduced. However, when this experiment was repeated and the physo-

stigmine response tested every 10 min, followed by replacement of the Mipafox, no reduction in the response to physostigmine had occurred in 80 min, illustrating the protection of the enzyme by physostigmine.

Comparisons of the effects produced by equimolar concentrations of dyflos and Mipafox are shown in Fig. 7. Two 14-day membranes from the same batch of eggs were used. Control responses are seen in the extreme left-hand panels. Dyflos

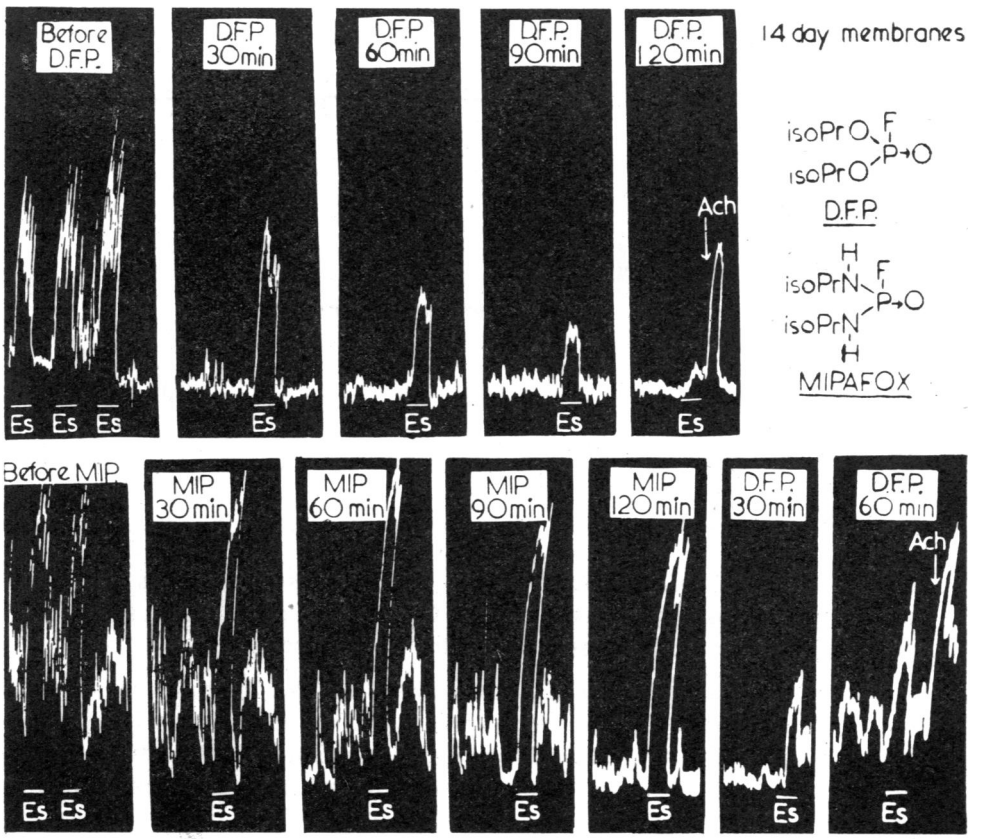

Fig. 7. Amniotic membranes, 14 day. Doses of physostigmine were 5 μg/ml. given for 2 min, and of acetylcholine 5 μg/ml. given for 1 min. Concentrations of dyflos and Mipafox used were 0.5 mg/ml. (2.7×10^{-3} M). These inhibitors were removed from the bath 5 min before testing the physostigmine response. The time in min shown at the top of the panels gives the duration of exposure to dyflos or Mipafox.

and Mipafox were used in a concentration of 2.7×10^{-3} M. The first amniotic membrane was treated with dyflos for 30 min at 37° C. At the end of this time it was washed; 5 min were then allowed for the tissues to equilibrate before the response to physostigmine was tested. After removal of the physostigmine the tissue was re-exposed to the same concentration of dyflos for a further 30 min, after which the testing procedure with physostigmine was repeated. Under these conditions 2 hr were required to abolish the physostigmine response, while the

membrane still remained sensitive to acetylcholine. On repeating this experiment on the second membrane with Mipafox, the response to physostigmine after 2 hr was unaffected. Subsequent replacement of Mipafox by dyflos reduced the physostigmine response in 30 min, and after 1 hr of exposure to dyflos an increase in tone of the muscle was seen.

The effect of calcium on the response to physostigmine is shown in Fig. 8. Control contractions to physostigmine of a spontaneously active preparation were obtained. The bathing solution was then changed to calcium-free Hanks' balanced salt solution

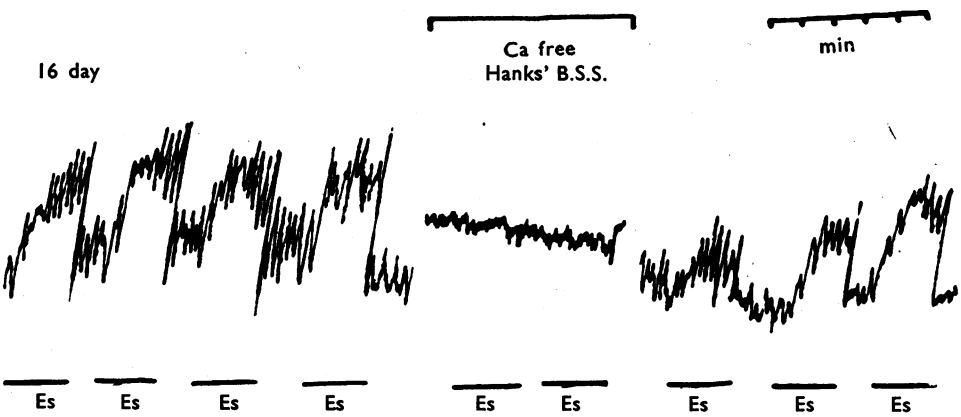

Fig. 8. Amniotic membrane, 16 day. Doses of physostigmine were 5 μg/ml. for 2 min.

containing 200 mg/l. of disodium edetate. Spontaneous contractions ceased and no response to physostigmine was observed. On replacing calcium both spontaneous contractions and the contractions to physostigmine reappeared.

Manometric determinations of cholinesterase. The Q_{CO_2} value (μl. CO_2/hr/g of tissue) for chick amnion tissue was low, about 300, when acetylcholine (0.03 M) was used as substrate. Dyflos and Mipafox at a concentration of 10^{-8} produced respectively 78% and 89.6% inhibition of amnion cholinesterase in 11-day membranes after previous incubation of the homogenate for 1 hr at 37° C. The combination of a low Q_{CO_2} value and the difficulty in obtaining adequate amounts of amniotic tissue made accurate determinations tedious. However, since amnion cholinesterase and hen plasma cholinesterase are identical (Blaber & Cuthbert, 1962) the latter was used in some experiments. The molar concentrations of physostigmine and neostigmine producing 50% inhibition of hen plasma cholinesterase were respectively 2.6×10^{-8} and 2.2×10^{-8}. The rate of hydrolysis of α-naphthyl acetate was 85% of the rate of acetylcholine hydrolysis at concentrations of 0.03 M using hen plasma as the source of enzyme.

Staining of cholinesterase and amniotic membranes for cholinesterase. The experiment was performed on freshly prepared hen plasma and 9-day amniotic membranes. One membrane suspended in Hanks' solution and one tube of 50% hen plasma in distilled water were incubated at 37° C for 1 hr and served as controls. A

second membrane and plasma sample were incubated for the same time with 10^{-2} M Mipafox, and a third membrane and plasma sample with 10^{-2} M dyflos. After 1 hr, 3 ml. aliquots of the plasma samples were mixed with 2 ml. of incubation medium containing α-naphthyl acetate. After 15 min further incubation at 37° C the tubes were photographed. The three amniotic membranes were placed in the incubation medium at 37° C for 90 min, then removed, washed in Hanks' solution, and photographed. Both the control plasma and membrane stained for cholinesterase. Neither of the treated samples of plasma were stained, but the Mipafox-treated membrane stained as deeply as the control (Fig. 9). Prolonged incubation, however, of amniotic membranes with Mipafox inhibited the staining reaction for cholin-

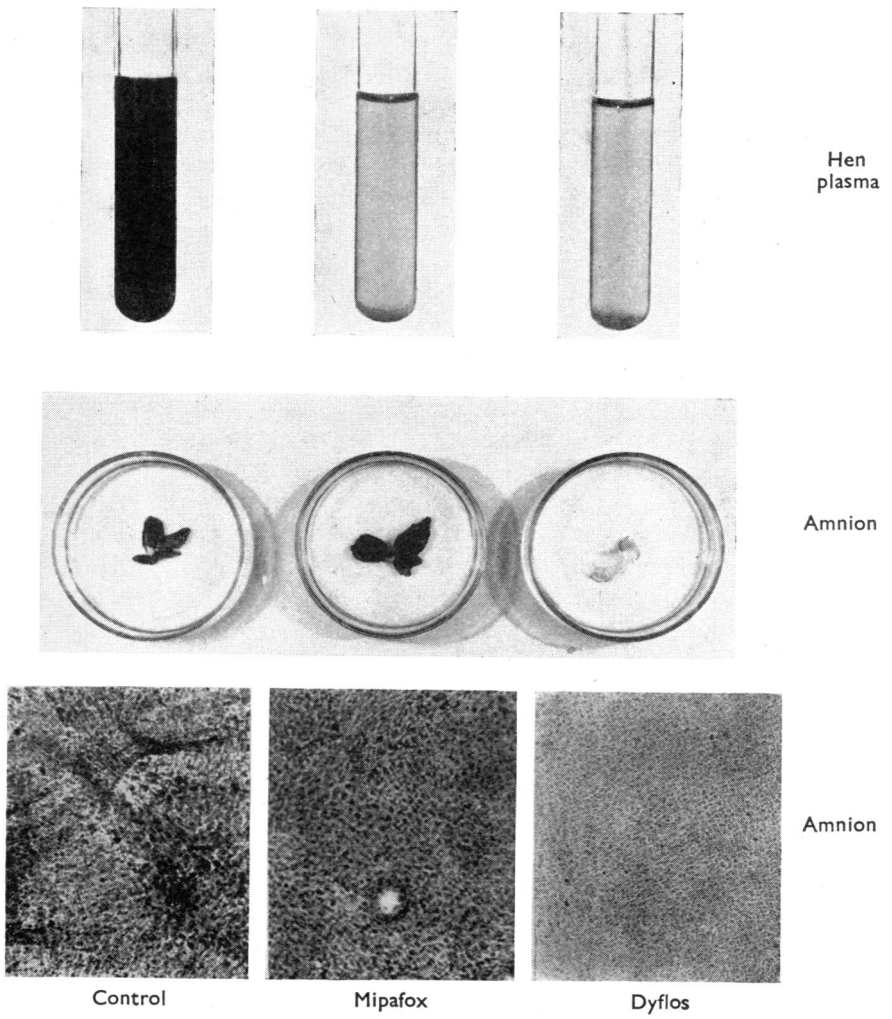

Fig. 9. Amniotic membranes, 9 day, and hen plasma stained for cholinesterase. Photomicrographs taken from tissues seen in the middle row. For explanation see text.

esterase. It was found that amnion tissue incubated for 2 hr with 10^{-2} M Mipafox at 37° C showed an intensity of staining comparable to tissue which had been incubated in 10^{-5} M dyflos under the same conditions.

DISCUSSION

Evans, Schild & Thesleff (1958) and Ferguson (1940) have previously reported the effects of acetylcholine on the chick amnion and the antagonism of acetylcholine by atropine. A pA_2 value of 8.8 for acetylcholine-atropine antagonism was obtained by Evans & Schild (1959) on the amnion, which agrees well with the value for other smooth-muscle tissues. Therefore the presence of a normal muscarinic type of acetylcholine receptor seems most likely. An acetylcholine-like substance was found (Cuthbert, 1960) in extracts prepared from amnion tissue, and Blaber & Cuthbert (1962) have recently characterized the cholinesterases in several avian tissues including the chick amnion. They found the cholinesterase of chick amnion to be a type of pseudocholinesterase intermediate in properties between those of the acetocholinesterase of hen brain and the butyrocholinesterase of most mammals; unpublished histochemical findings have shown that the enzyme is confined to the smooth-muscle layer, being absent from the endothelial layer of cells. It seems likely that the effect of physostigmine is due to the accumulation of endogenously produced acetylcholine caused by the inhibition of amnion cholinesterase. Other evidence presented here supports this idea.

The effect of physostigmine parallels to some degree the activity shown by the amnion *in ovo*. Spontaneous contractions in the egg appear first after about 4 days' incubation; they increase to a maximum after about 10 days and then decline. Spontaneous contractions are rarely seen after the 15th day of incubation (Romanoff, 1952). Much evidence has been produced to show that acetylcholine is responsible for automaticity in the heart and cilia (see review by Burn, 1954), and this may also be true of the amnion. A cholinesterase inhibitor would therefore be expected to produce its maximal effects when endogenous acetylcholine production is high but to be without effect when this has stopped. This is true for the effects of physostigmine on the chick amnion, except when activity and tone are maximal, at which time the tissue may be supposed to be responding maximally to its acetylcholine. Also the response to physostigmine reappears in inactive preparations in which spontaneity has been restored with acetylcholine or choline. The failure of atropine to block the response to eserine is unusual, since the block of applied acetylcholine is complete and prolonged. It is well known that atropine fails to antagonize the effects of transmitter release from some cholinergic nerves, whereas externally applied acetylcholine is readily blocked. It has been suggested (Dale & Gaddum, 1930) that the release occurs in such close proximity to the receptor that atropine antagonism cannot occur. A similar hypothesis could be applicable to the chick amnion. It is interesting that the older literature cites examples of nerve fibres penetrating muscle cells, suggesting an intracellular site of action of the transmitter (Lawrentjew, 1926a, b; Hill, 1927). The response to physostigmine is extremely rapid, usually appearing a few seconds after addition

of the drug, whereas on the ileum preparations the response to physostigmine appears far more slowly. It is suggested that this is due to the ease of penetration in a tissue in which a monocellular layer of muscle fibres is exposed.

The difference between physostigmine and neostigmine may be due to the latter possessing a quaternary ammonium ion which does not easily penetrate the cell membrane, as both compounds are equally effective in inhibiting the isolated enzyme. This implies that the functional cholinesterase in this tissue is intracellular. In neuromuscular preparations such as the sciatic gastrocnemius of the cat where the enzyme of the end-plate is exposed, neostigmine is the more effective in increasing twitch height, suggesting that the effective concentration of the tertiary physostigmine is quickly removed by absorption through cell membranes.

Relatively high concentrations of dyflos were required to produce an effect on the chick amnion compared with the low concentrations required to inhibit the enzyme. This may be due to its difficulty in penetrating the cell membrane, or to its removal after penetration by partition into lipid materials. The variable effects seen with dyflos on the isolated amnion are similar to those described by Koelle, Koelle & Friedenwald (1950) with the ileum of the cat. The effects of dyflos on the amnion are never so dramatic as those of physostigmine. It appears that a rapid, almost complete inactivation of cholinesterase is necessary for acetylcholine accumulation. With prolonged exposure to dyflos it appears that the gradually reducing amount of enzyme is still able to prevent acetylcholine accumulation. Some part of the reduction in the physostigmine response after dyflos treatment might be a consequence of reduced synthesis of acetylcholine. However, experiments in which physostigmine was given at intervals over long periods have shown only a slight reduction in response, indicating only a slight fall-off in acetylcholine synthesis. The immediate prolonged stimulation with high doses of dyflos is probably due to an immediate reversible inhibition of enzyme.

The low potency of Mipafox compared with dyflos is difficult to understand. Although it is more rapidly hydrolysed, 10^{-8} M Mipafox is still able to cause 90% inhibition of free cholinesterase after incubation for 1 hr at 37° C, whereas 10^{-2} M Mipafox for 1 hr at 37° C causes little or no inhibition of cholinesterase staining in the amnion as compared with the control.

The results obtained with physostigmine and neostigmine and with dyflos and Mipafox are consistent with the view that the functional cholinesterase of the chick amnion is intracellular, and that the effects produced are due to acetylcholine accumulation. The failure of high concentrations of atropine to block the physostigmine response suggests the accumulated acetylcholine is not acting at the muscarinic site, presumably at the cell membrane.

Calcium ions are necessary for both spontaneous contractions and for the effect of physostigmine in the amnion, and it is suggested that the intracellular acetylcholine may act by releasing calcium from a bound form within the cell. Lüttgau & Niedergerke (1958) using the frog heart concluded that an increase in the calcium concentration at sites within the cell resulted in contraction. In skeletal muscle intracellularly applied calcium causes local muscular shortening (Niedergerke, 1955).

In several crustacean muscle fibres, Hoyle & Wiersma (1958a) envisaged a direct effect of transmitter on the excitation-contraction coupling mechanism, electrical events at the membrane being secondary. In a subsequent paper they suggested that calcium may be intimately involved in excitation-contraction coupling (Hoyle & Wiersma, 1958b). Intracellular application of acetylcholine with simultaneous electrical recording in the amnion would be a direct method of tackling this problem, and such experiments are in progress.

Financial assistance from Pfizer Ltd. during the tenure of this work is gratefully acknowledged. I am also indebted for a gift of brentamine fast red T.R. salt from I.C.I.

REFERENCES

BLABER, L. C. & CUTHBERT, A. W. (1962). Cholinesterases in the domestic fowl and the specificity of some reversible inhibitors. *Biochem. Pharmacol.*, **2**, 113–124.

BÜLBRING, E. & BURN, J. H. (1949). Action of acetylcholine on rabbit auricles in relation to acetylcholine synthesis. *J. Physiol. (Lond.)*, **108**, 508–524.

BÜLBRING, E., BURN, J. H. & SHELLEY, H. (1953). Acetylcholine and ciliary movement in the gill plates of Mytilus. *Proc. roy. Soc. Ser. B.*, **141**, 445–466.

BURN, J. H. (1954). Acetylcholine as a local hormone for ciliary movement and the heart. *Pharmacol. Rev.*, **6**, 107–112.

COMLINE, R. E. (1946). Synthesis of acetylcholine by non-nervous tissue. *J. Physiol. (Lond.)*, **105**, 6–7*P*.

CUTHBERT, A. W. (1960). Some studies on chick amnion. *Brit. J. Pharmacol.*, **15**, 362.

DALE, H. H. & GADDUM, J. H. (1930). Reactions of denervated voluntary muscle and their bearing on the mode of action of parasympathetic and related nerves. *J. Physiol. (Lond.)*, **70**, 109–144.

EVANS, D. H. L. & SCHILD, H. O. (1959). Unpublished results cited by ARUNLAKSHANA, O. & SCHILD, H. O. Some quantitative uses of drug antagonists. *Brit. J. Pharmacol.*, **14**, 48–58.

EVANS, D. H. L., SCHILD, H. O. & THESLEFF, S. (1958). Effects of drugs on depolarized plain muscle. *J. Physiol. (Lond.)*, **143**, 474–485.

FELDBERG, W. S. (1950). On the origin and function of the acetylcholine in the intestinal wall. *Proc. roy. Soc. Ser. B.*, **137**, 285–292.

FELDBERG, W. & LIN, R. C. Y. (1950). Synthesis of acetylcholine in the wall of the digestive tract. *J. Physiol. (Lond.)*, **111**, 96–119.

FERGUSON, J. (1940). A study of the nerve-free smooth muscle of the amnion of the chick. *Amer. J. Physiol.*, **131**, 524–534.

FUJINO, M., MATSUSHIMA, T., MUROYA, T., YABU, H., YAMAGUCHI, S. & TAKAHASHI, M. (1960). Effect of electrical stimulation on a muscle fibre model in the presence of adenosine triphosphate. *Nature (Lond.)*, **186**, 318–320.

HILL, C. J. (1927). A contribution to our knowledge of the enteric plexuses. *Phil. Trans. Ser. B.*, **215**, 355–387.

HOYLE, G. & WIERSMA, C. A. G. (1958a). Inhibition at neuromuscular junctions in crustacea. *J. Physiol. (Lond.)*, **143**, 426–440.

HOYLE, G. & WIERSMA, C. A. G. (1958b). Coupling of membrane potential to contraction in crustacean muscles. *J. Physiol. (Lond.)*, **143**, 441–453.

KOELLE, G. B., KOELLE, E. S. & FRIEDENWALD, J. S. (1950). The effect of inhibition of specific and non-specific cholinesterase on the motility of the isolated ileum. *J. Pharmacol. exp. Ther.*, **100**, 180–191.

LAWRENTJEW, B. J. (1926a). Über das Chondriom der Grandryschen Körperchen. *Z. mikr.-anat. Forsch.*, **6**, 241–255.

LAWRENTJEW, B. J. (1926b). Über die Verbreitung der nervösen Elemente (einschliesslich der "interstitiellen Zellen" Cajals) in der glatten Muskulatur, ihre Endigungsweise in den glatten Muskelzellen. *Z. mikr.-anat. Forsch.*, **6**, 467–488.

LEWIS, P. R. (1958). A simultaneous coupling azo dye technique suitable for whole mounts. *Quart. J. micr. Sci.*, **99**, 67–72.

LÜTTGAU, H. C. & NIEDERGERKE, R. (1958). The antagonism between Ca and Na ions on the frog's heart. *J. Physiol. (Lond.)*, **143**, 486–505.

NIEDERGERKE, R. (1955). Local muscular shortening by intracellularly applied calcium. *J. Physiol. (Lond.)*, **128**, 12–13*P*.

ROMANOFF, A. L. (1952). Membrane growth and function. *Ann. N.Y. Acad. Sci.*, **55**, 288–301.