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AN ACETYLCHOLINE-LIKE SUBSTANCE AND CHOLINESTERASE IN THE SMOOTH MUSCLE OF THE CHICK AMNION

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Previous work has indicated that the spontaneous beat of the chick amnion may be due to the formation and release of acetylcholine in the smooth muscle cells of this organ (Cuthbert, 1962). In this respect the amnion, which contains no nerves, may be like other non-nervous tissue such as the cilia of the gill plates of *Mytilus*, for which it has been claimed that acetylcholine formed within the tissue controls the frequency of movement (Burn, 1954).

In the present experiments on the chick amnion this idea has been tested by analysing extracts of the tissue in order to determine whether they contain any acetylcholine-like substance, by histochemical investigation to observe the distribution of cholinesterases in the tissue and by an additional study of the isolated beating amnion.

METHODS

Amniotic membranes were dissected from hens' eggs incubated for periods of 4–15 days at 37° C. The eggs were not turned during incubation.

Tests for the presence of acetylcholine-like substances in chick amnion extracts

Preparation of extracts. The principles suggested by MacIntosh & Perry (1950) for the preparation of extracts for assay of acetylcholine were followed. The amniotic membranes were dissected in a cold room, at 5° C, with chilled instruments. The membranes were quickly washed by dipping in Hanks's (1946) balanced salt solution and dried by dragging over the surface of a Petri dish until they no longer left a wet trail. They were placed in a graduated centrifuge tube containing 2 ml. of ice-cold 10% trichloroacetic acid and about 0·2 g of A.R. grade sand. The tube of trichloroacetic acid and sand had been previously weighed. When all the membranes had been transferred to the tube it was reweighed. The tissues were ground in the trichloroacetic acid and the tube was allowed to stand for 90 min at room temperature. At the end of this time the tube was centrifuged at 3000 rev/min for 10 min, and the clear supernatant removed by pipette and placed in a stoppered cylinder. The trichloroacetic acid was removed by shaking four times with four volumes of water-saturated ether, and residual ether was removed by aeration. The extracts thus prepared were stored at 0° C until assayed. With care the volume loss did not exceed 0·2 ml. The amount of tissue

available was between 0·1 and 0·5 g, but the volume of trichloroacetic acid was kept at 2 ml. to avoid manipulative losses.

Biological examination of extracts. Extracts were tested on the blood-pressure response of rats anaesthetized with urethane (40 mg/100 g) and sodium pentobarbitone (3 mg/100 g), as described by Straughan (1958). Neostigmine methyl sulphate (1 μg/ml.) in normal saline was used to wash in the doses and to increase the sensitivity to acetylcholine. The effect of extracts on the dorsal muscle of the leech suspended in Locke's solution at room temperature was also tested. The Locke's solution was diluted five parts to seven parts with water and contained 4 μg/ml. of eserine. In a few cases the blood-pressure response of the eviscerated cat, in the presence of neostigmine methyl sulphate, was also used.

Histochemical staining for cholinesterase

Two methods were used to demonstrate the presence of cholinesterase.

Thiocholine method. The modified method of Koelle (1951) was used, to which an extra stage was added. This consisted of freezing the amniotic membranes, spread on slides, for 1 hr at − 10° C before placing in the incubation mixture at 37° C. Without this procedure the staining was poor, and it is thought that freezing ruptured the cell membranes and so allowed the substrates to penetrate the cell. Acetylthiocholine and butyrylthiocholine were used as substrates. Inclusion of eserine ($3{\cdot}6 \times 10^{-5}$ M) in the incubation solutions prevented the staining with this method.

Diazo-coupling method. A method based on that described by Lewis (1958) was used in which the first stage (soaking of the tissue in the diazonium salt solution) was omitted. The amniotic membranes were spread on slides and placed in the incubation mixture at 37° C. The incubation mixture was composed as follows: 0·2 M Tris buffer, 1·5 ml.; N-HCl, 0·15 ml.; Brentamine fast red T.R. salt, 25 mg; α-naphthyl acetate 10 mg; water, 18·5 ml. These ingredients were vigorously shaken for 30 sec and filtered immediately before use. Control preparations, in which the cholinesterase was inhibited, were obtained by immersing the slides in 10^{-2} M DFP in Hanks's balanced salt solution for 1 hr at 37° C before staining. Eserine is not a suitable inhibitor for use with this method, as it reacts with the incubation solution to give a red-brown colour, similar to that of the stained material. Since 10^{-8} M DFP causes 78 % inhibition of esterases present in a homogenate of amnion tissue, the high concentration required to inhibit the staining reaction is thought to be due to the failure of DFP to penetrate the tissue. This idea is supported by the high concentrations of DFP required to reduce the response of the isolated amnion to eserine (Cuthbert, 1962). But the possibility that part of the staining reaction in the amnion with α-naphthylacetate as substrate was due to esterases other than cholinesterases cannot be excluded.

Method for determining the effects of eserine on the isolated chick amnion

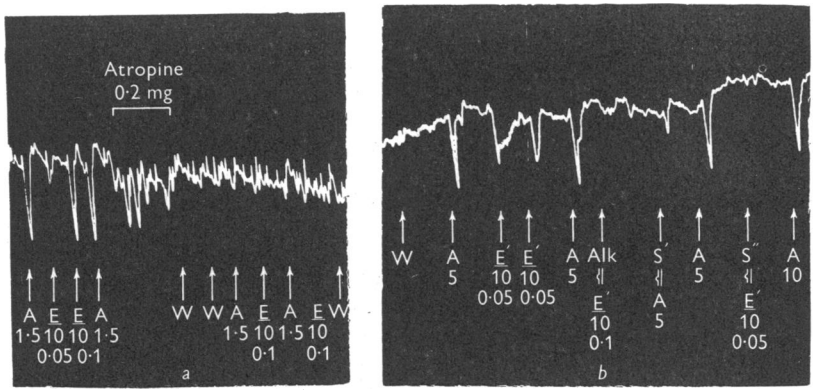
The amniotic membranes were suspended in an isolated organ bath in Hanks's (1946) balanced salt solution at 37° C and gassed with air. Isotonic contractions of the muscle were recorded on a smoked drum as described previously (Cuthbert, 1962).

Reagents. The materials used were acetylcholine chloride, acetylthiocholine iodide, butyrylthiocholine iodide, α-naphthylacetate, atropine sulphate, D-tubocurarine chloride, neostigmine methyl sulphate, eserine salicylate, di-*iso*propyl phosphorofluoridate, Brentamine fast red TR salt, mepyramine maleate and papaverine hydrochloride. Unless otherwise stated concentrations refer to the bases. The composition of Hanks's balanced salt solution was: (g/l.) NaCl, 8·0; KCl, 0·4; $CaCl_2$, 0·14; $MgSO_4.7H_2O$, 0·10; $MgCl_2.6H_2O$, 0·10; $Na_2HPO_4.2H_2O$, 0·06; KH_2PO_4, 0·06; glucose, 1·0 and $NaHCO_3$, 0·35: this solution was gassed with air before use.

RESULTS

Examination of amnion extracts for acetylcholine-like substances

A substance resembling acetylcholine has been found in extracts of chick amniotic membranes. Extracts were prepared from membranes of between 8 and 10 days incubation, since maximal spontaneous activity is seen at this time (Romanoff, 1952). It has not been possible to rule out the presence of an ester other than the acetyl derivative in these extracts. The extracts were of small volume (2 ml.) and contained only small amounts of active material. They also appeared to be unstable even when stored at



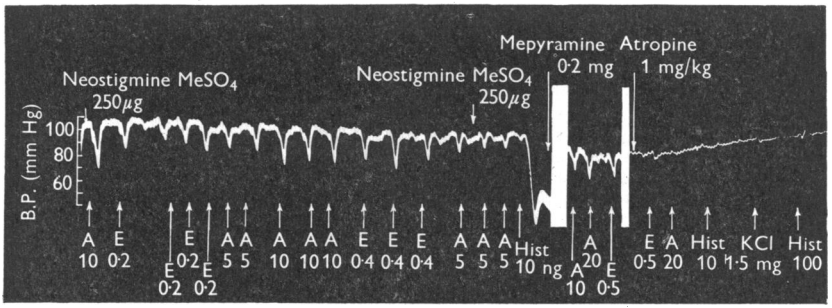
Text-fig. 1*a*. The effect of atropine on the depressor response to amnion extract (E), and to acetylcholine (A) on the blood pressure of the rat. Extract prepared from 9-day membranes and diluted 1 to 10; doses of diluted extract in ml. and of acetylcholine in ng. 1 ml. of extract was equivalent to 150 ng acetylcholine (representing 0·675 μg/g wet wt. of tissue). At W 0·2 ml. saline was given.

b. The effect of warming with alkali, and of incubation with horse serum, on the activity of an 8-day amnion extract E′ assayed on the blood pressure response of the rat. Doses of acetylcholine (A) in ng, and of amnion extract (diluted 1 to 10) in ml. At Alk the effect of an amount, equivalent to 0·1 ml., of diluted extract which had been warmed with alkali and neutralized is shown. The effects of 5 ng acetylcholine and of 0·05 ml. diluted extract incubated with horse serum are shown at S′ and S″, respectively.

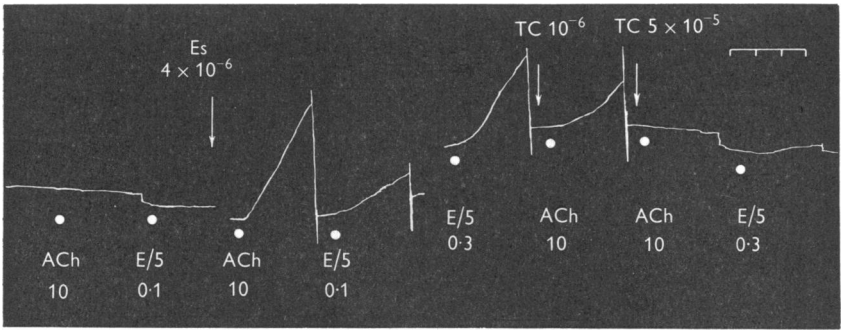
0° C. In a typical experiment with 9-day eggs, 0·44 g tissue (representing 10 amniotic membranes) was extracted to give a final volume of 2 ml. When tested on the rat blood pressure the extract contained 300 ng/ml. of active material, calculated as acetylcholine. This represented a tissue concentration of 1·35 μg/g wet weight of tissue. After storage for a further 2 days at 0° C the extract contained 150 ng/ml. calculated as acetylcholine, when again tested on the rat blood pressure.

Several qualitative tests have been applied to the extracts. Atropine blocks the fall in blood pressure caused by the extracts or by acetylcholine

in the rat (Text-fig. 1*a*) and the cat (Text-fig. 2). Mepyramine did not affect the depressor response in the cat and no response was obtained to potassium in amounts liable to be present in extracts (Text-fig. 2). Tubocurarine was able to block the contraction of the leech muscle in response to both extracts and to acetylcholine. The responses of the leech muscle to the extracts and to acetylcholine were both potentiated by eserine (Text-fig. 3).



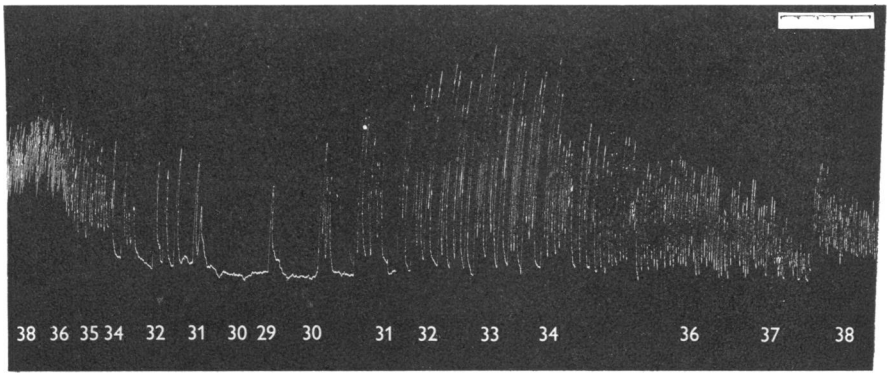
Text-fig. 2. The effect of an 8-day amnion extract (E) and of acetylcholine (A) on the blood pressure of the eviscerated cat. Doses of acetylcholine in ng and of extract in ml. Each 1 ml. of extract was equivalent to 25 ng calculated as acetylcholine representing a tissue concentration of 0·16 μg/g. (This extract was stored for 1 week before assay.)



Text-fig. 3. Contractions of the dorsal muscle of the leech to amnion extract (E) and to acetylcholine (ACh). Doses of acetylcholine given as ng and of extract (diluted 1:5) given as ml. At Es eserine 4 μg/ml. was added to the modified Locke's solution. At TC D-tubocurarine chloride was added to the bath in the concentrations indicated. Time marker, minutes.

When 0·2 ml. of extract was warmed with 1 drop of N/3-NaOH for 30 sec, and subsequently neutralized, the activity was destroyed. Similarly, extracts (diluted 1:10) incubated at 37° C for 5 min with an equal volume of horse serum lost their activity, as did acetylcholine solutions of comparable activity. The dose–response curves for amnion extract and acetyl-

choline, plotted from Text-fig. 2 (for responses between the two doses of neostigmine), did not deviate significantly from parallel.



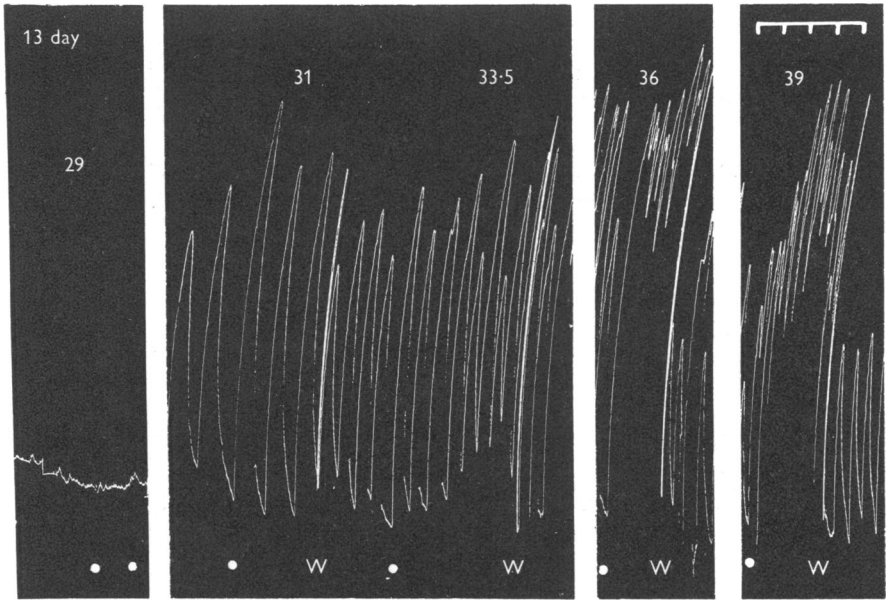
Text-fig. 4. Spontaneous contractions of a 12-day chick amnion in an isolated organ bath. Figures indicate temperature (° C). Time marker, minutes.

The effect of drugs and temperature on the spontaneous activity of the chick amnion

The spontaneous contractions shown by the isolated chick amnion in Hanks's balanced salt solution were dependent on temperature. Text-fig. 4 shows the effect of raising and lowering the temperature between 29 and 38° C. On lowering the temperature from 38° C to about 36° C the contraction rate diminished and tone declined. At 30° C contractions had almost ceased, only one being seen during 6 min. On rewarming the frequency of spontaneous contractions returned to a value approaching nearly its former level. The maximal amplitude was usually seen at a lower temperature than that at which frequency was maximal. It was noted in many preparations that at 38° C there is a rough inverse proportionality between rate and amplitude, that is, the higher the frequency the smaller the amplitude. The response of the chick amnion to eserine also showed a gradation with temperature. This is illustrated in Text-fig. 5. Initially no spontaneous contractions were seen and eserine (2·5 μg/ml.) had no effect. A slight but definite increase in tone after eserine was seen at 33·5° C. At 36 and 39° C eserine caused an immediate increase in tone and frequency of the spontaneously contracting chick amnion.

It was of interest to test whether eserine caused a contraction of the amnion in preparations in which the cell membranes were depolarized. Evans, Schild & Thesleff (1958) found that when the chick amnion was immersed in Ringer's solutions in which NaCl was replaced by K_2SO_4, and $NaHCO_3$ by $KHCO_3$, the membrane potential fell to zero. Unfortunately when amnions are immersed in such a modified solution at 38° C the tissue

goes into a contracture which is maintained for several hours. Under these conditions eserine had no further stimulating effect. However, if papaverine was also added to the K_2SO_4-Hanks's solution, relaxation occurred after the initial contracture. This drug does not affect the membrane potential of other excitable cells (Szekeres & Vaughan Williams, 1962),

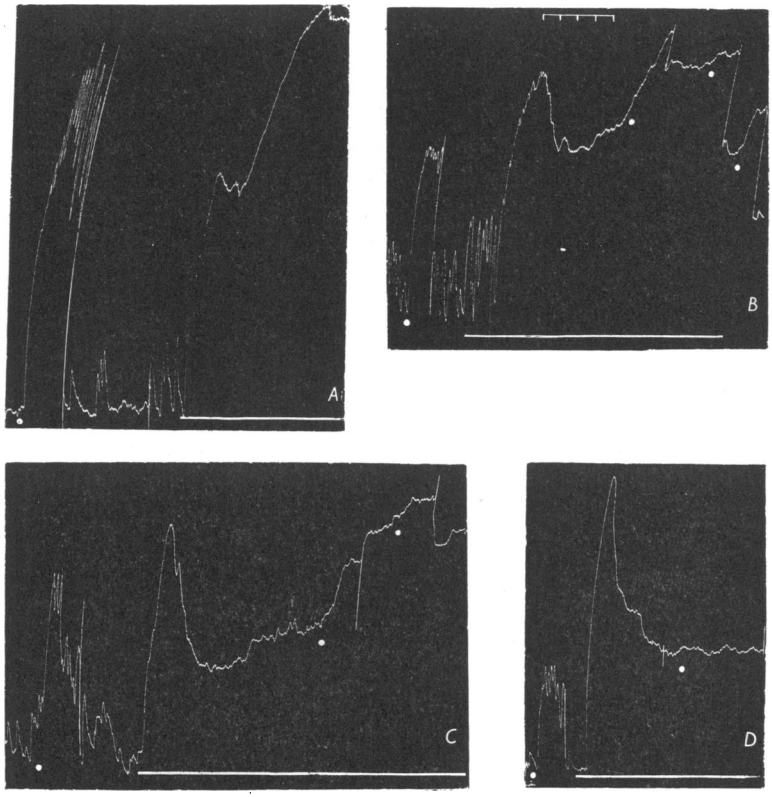


Text-fig. 5. Response of a 13-day chick amnion to eserine. At the white dots eserine, 2·5 μg/ml., was given. W indicates when the bath fluid was replaced with fresh solution. Figures indicate temperature (° C).

and it has been assumed that it similarly has no effect on the amnion. The results of one series of experiments are shown in Text-fig. 6. Experiment *A* was performed on a 13-day isolated amnion. The preparation responded to 2·5 μg eserine/ml. with an increase in tone and frequency of contraction. When the Hanks's solution was changed to K_2SO_4-Hanks's solution there was a sudden increase in tone which had not declined 2·5 hr later.

Experiments *B*, *C* and *D* shown in Text-fig. 6 were performed on 15-day membranes. In *B* a response was obtained to 2·5 μg eserine/ml. The preparation was then immersed in K_2SO_4-Hanks's solution containing 1 μg papaverine hydrochloride/ml. After the initial contracture the tone was reduced to 68 % of the peak level, but it tended to increase slowly after this. Eserine (2·5 μg/ml.) caused a rapid increase in tone to a level greater than that of the initial contracture. After removal of the eserine only incomplete relaxation was obtained. A second application of eserine had a

very slight effect. In *C* the K_2SO_4-Hanks's solution contained 2 μg papaverine hydrochloride/ml. The initial contracture was followed by a relaxation to 40 % of the peak height. Eserine again caused a sudden increase in tone, the first application being the most effective. Again incomplete

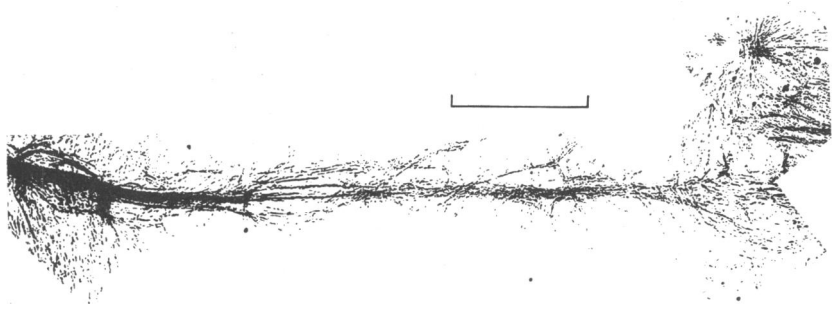


Text-fig. 6. The effects of eserine on four amniotic membranes immersed in potassium-rich solutions. At the white dots eserine was added to produce a concentration of 2·5 μg/ml. in *A* and *B* and 5 μg/ml. in *C* and *D*. The solid white line in each panel indicates the periods when preparations were in K_2SO_4-Hanks's solution. The K_2SO_4-Hanks's solution contained papaverine hydrochloride 0, 1, 2 and 5 μg/ml. in experiments *A*, *B*, *C* and *D*, respectively.

relaxation was seen after removal of the eserine. Papaverine hydrochloride, at a concentration of 5 μg/ml., was contained in the K_2SO_4-Hanks's solution in experiment *D*. The preparation relaxed to 37 % of the peak height of contracture and there was no tendency for a subsequent increase in tone. Eserine had no effect at this concentration of papaverine. Thus maximal relaxation under these conditions was given by 2 μg/ml. of papaverine, higher concentrations causing inhibition of the eserine response.

The results of histochemical staining for cholinesterase in the chick amnion

The amniotic membrane of the chick showed positive staining for cholinesterase both by the method of Koelle (1951) and the method of Lewis (1958). In the former method the density of staining was approximately the same whether acetylthiocholine or butyrylthiocholine was used as substrate, and inclusion of eserine ($3{\cdot}6 \times 10^{-5}$M) in the incubation solution prevented the staining reaction (Pl. 1, *a–d*). In the Lewis method, in which α-naphthyl acetate was used as substrate, previous incubation of the tissue for 1 hr at 37° C in 10^{-2}M DFP inhibited the staining reaction (Pl. 2*a*, *b*). Essentially the same picture was seen with both methods of staining and with all three substrates, suggesting that the same enzyme,



Text-fig. 7. Photomontage from eight fields of a 9-day chick amnion incubated with butyrylthiocholine for 6 hr at 37° C. Scale = 1 mm. The small spherical, very dense objects seen in this figure were particles of copper sulphide which escaped filtration in the preparation of the copper-sulphide-saturated dehydrating and clearing solutions.

namely cholinesterase, was detected in each case. All the smooth-muscle cells showed the staining reaction, but it was not possible to detect any cellular localization under high-power magnification. The whole of the sarcoplasm of the individual cells appeared to be stained. The single layer of endothelial cells which is attached to the single layer of smooth-muscle cells contained very little, or no cholinesterase. In Pl. 2*c*, *d* the torn edge of amniotic membranes stained by Lewis's (1958) method are shown. The upper muscle layer is stained, whereas the underlying endothelial layer appears unstained. It was evident from all the preparations examined that the 'cross' figures, which are characteristic of amnion musculature, are more deeply stained than the surrounding tissue (Pls. 1, 2; Text-fig. 7). In amnions from eggs incubated for 6 days the staining reaction was poor, and definite staining was seen only at the 'cross' figures (Pl. 1*e*). As the 'cross' figures develop in the amnion they become interwoven and interconnected with bands of muscle tissue. A complex of interwoven 'cross'

figures is seen in the left-hand panel of Pl. 2*a*, *b*. A region containing 'cross' figures with a connecting band of muscle tissue stained for cholinesterase is seen in Text-fig. 7. This region was about $4\frac{1}{2}$ mm in length. Complexes of 'cross' figures are seen at both extremities of the bands and minor 'cross' figures are seen along the length of the band. The smooth-muscle tissue surrounding the band was only poorly stained.

DISCUSSION

The results presented in the first part of this paper indicate that the chick amnion contains a substance indistinguishable from acetylcholine in a number of qualitative tests. The active substance resembled acetylcholine in that its effects on blood pressure were prevented by atropine, and that its effect on the leech muscle was blocked by D-tubocurarine. Further, the effects due to this substance were potentiated by eserine and prevented by incubation of the extracts with horse serum or by warming with alkali. Such tests confirm the presence of a choline ester but do not distinguish between different esters. It was not possible to perform parallel assays on different preparations with the same extract, for the reasons given previously; however, dose–response curves to extracts and to acetylcholine were parallel. It seems probable that the substance concerned was, in fact, acetylcholine. The active principle in the extracts must have been derived from either the smooth-muscle layer or endothelial layer, as the amnion is free of nervous elements.

Possibly this acetylcholine-like substance is responsible for maintaining the spontaneous rhythm of the amnion muscle. A similar role for acetylcholine has been postulated for the heart (Burn, 1950, 1954) and for the intestine (Feldberg & Lin, 1950). Evidence presented in a previous paper (Cuthbert, 1962) suggested that the increase in activity of the amnion caused by the anticholinesterase drug eserine was the result of the accumulation of an acetylcholine-like substance in the tissue. Evidence for this was based on the failure of eserine to affect the beat of amnions in which the cholinesterase had been inactivated by an irreversible organophosphorus anticholinesterase drug, or in which the ability to contract spontaneously had been lost.

In the present work the effect of temperature on the response was investigated. There was a close parallel between the effects of temperature on the spontaneous activity and on the response to eserine. If the response to eserine depends upon acetylcholine accumulation, a procedure which reduces the synthesis of this substance would be expected to reduce the eserine response.

The onset and direction of the spontaneous contractions of the amnion *in ovo* is correlated with the development and distribution of 'cross'

figures. Revoutskaia (1944) stated that the development of the 'cross' figures occurred in three stages at 4, 6 and 7 days of incubation. Contractions of the amnion appear first at 4 days, which corresponds to the formation of the earliest group of 'cross' figures. The 'cross' figures are most numerous between days 7 and 9, maximal spontaneous activity being shown at about this time (Romanoff, 1952). The greatest density of 'cross' figures occurs in the head-fold region with a smaller concentration in the tail fold (Pierce, 1933; Bautzman & Schroeder, 1953). Pierce describes contractions commencing at the head fold and proceeding to the tail, with occasional waves arising in the tail and proceeding a short way cephalad. From 14 days on, 1 or 2 'cross' figures are seen compared with 10–12/mm^2 at 5 days (Clements & Ferguson, 1951) and spontaneous contractions have almost ceased.

Results presented here show that amnion cholinesterase is concentrated in the 'cross' figures and interconnecting bundles of muscle fibres and is present to a much less extent in the rest of the smooth muscle of the amnion. It does not necessarily follow that acetylcholine has a function in tissues in which cholinesterase is concentrated, but such a relation suggests itself. The cholinesterase content of the chick intestine in culture increases in the presence of acetylcholine (Jones, Featherstone & Bonting, 1956); thus local concentrations of the enzyme may result from accumulation of the substrate. The enzymes of the amnion and chick intestine are similar (Cuthbert, unpublished results).

It is suggested, therefore, that the 'cross' figures may represent sites at which spontaneous contractions are initiated in response to an intracellular accumulation of an acetylcholine-like substance. Cells in which the critical concentration of acetylcholine is reached most quickly would act as pace-makers. The interconnecting bands may be tissue in which rapid conduction occurs, so that many pace-makers may discharge almost simultaneously. This would increase the safety factor, so that contractions initiated in one region would spread over the whole amniotic sac.

An intracellular site of action for the endogenous acetylcholine-like material in the amnion has been suggested (Cuthbert, 1962). The hypothesis was based on the failure of atropine to block the response to eserine, although externally applied acetylcholine was readily antagonized. Furthermore, anticholinesterase drugs which failed to penetrate the cell membrane did not stimulate the amnion. As the response to eserine was dependent on calcium it was suggested that the intracellular accumulation of an acetylcholine-like material freed calcium from a bound form within the cell, so that it then directly affected the contractile mechanism.

Eserine therefore would be expected to produce a contraction of the amnion even when the cell membranes were depolarized. It has been shown

that small but definite effects of eserine occur in potassium-rich solutions. Unfortunately papaverine had to be used to relax the muscle in such solutions, and it is not known if this substance affects the membrane potential. Papaverine does not, however, affect the membrane potential of cardiac muscle (Szekeres & Vaughan Williams, 1962).

The acetylcholine-like substance in the amnion may be of importance in increasing excitability of the muscle cells in the bundles connecting 'cross' figures, and so increase the safety factor for conduction.

SUMMARY

1. A substance, indistinguishable from acetylcholine in several qualitative tests, has been found in extracts of chick amniotic membranes.

2. The frequency of spontaneous contractions and the responses to eserine of the isolated chick amnion were found to be dependent on the temperature.

3. Eserine is able to cause a contraction of the isolated chick amnion in K_2SO_4-Hanks's solution. This may be due to the intracellular accumulation of an acetylcholine-like substance, which activates the contractile mechanism without involving the muscle cell membrane.

4. The localization of cholinesterase in the amnion has been demonstrated by histochemical procedures in which three different substrates (acetylthiocholine, butyrylthiocholine and α-naphthylacetate) were used. Similar staining resulted with all three substrates.

5. 'The 'cross' figures and interconnecting bands of smooth muscle were most deeply stained for cholinesterase. It is suggested that the 'cross' figures are the pace-makers in which contractions are initiated.

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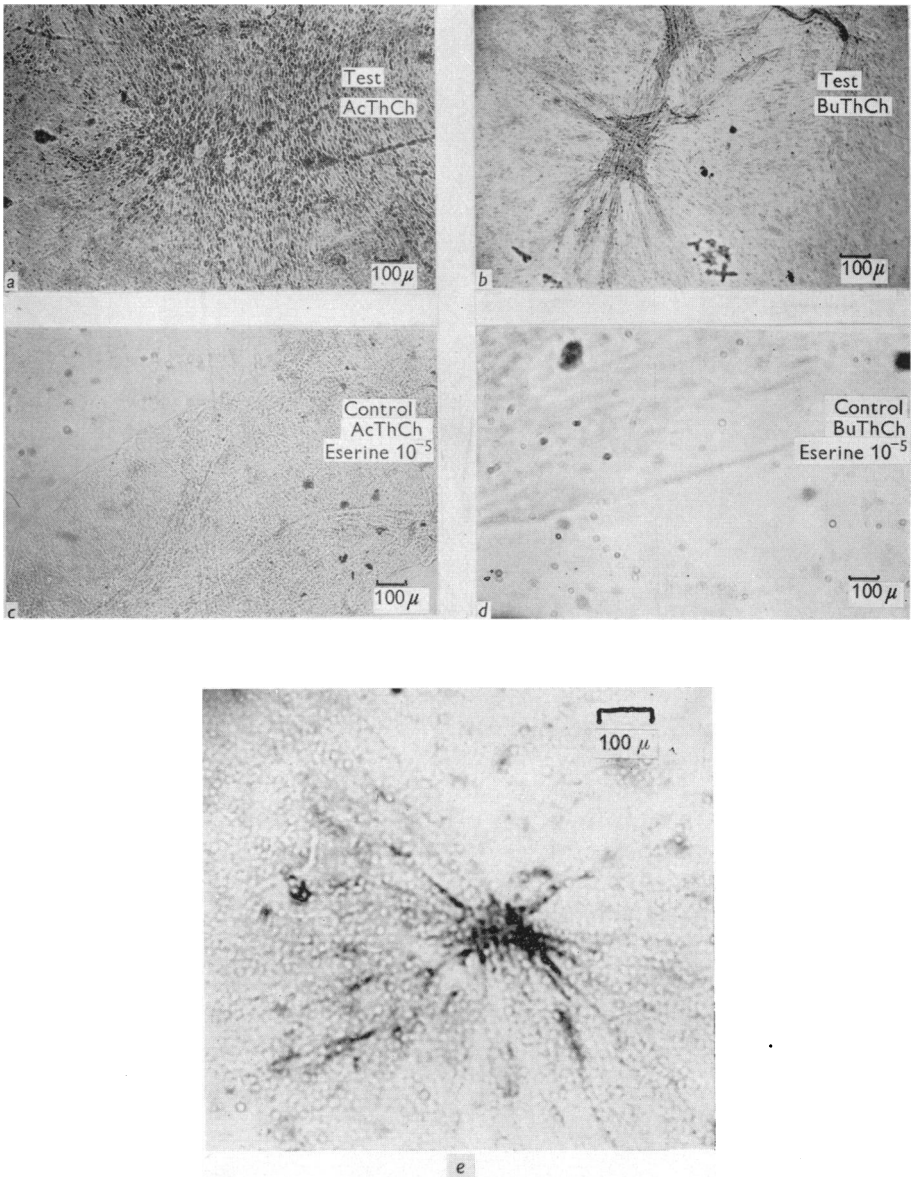
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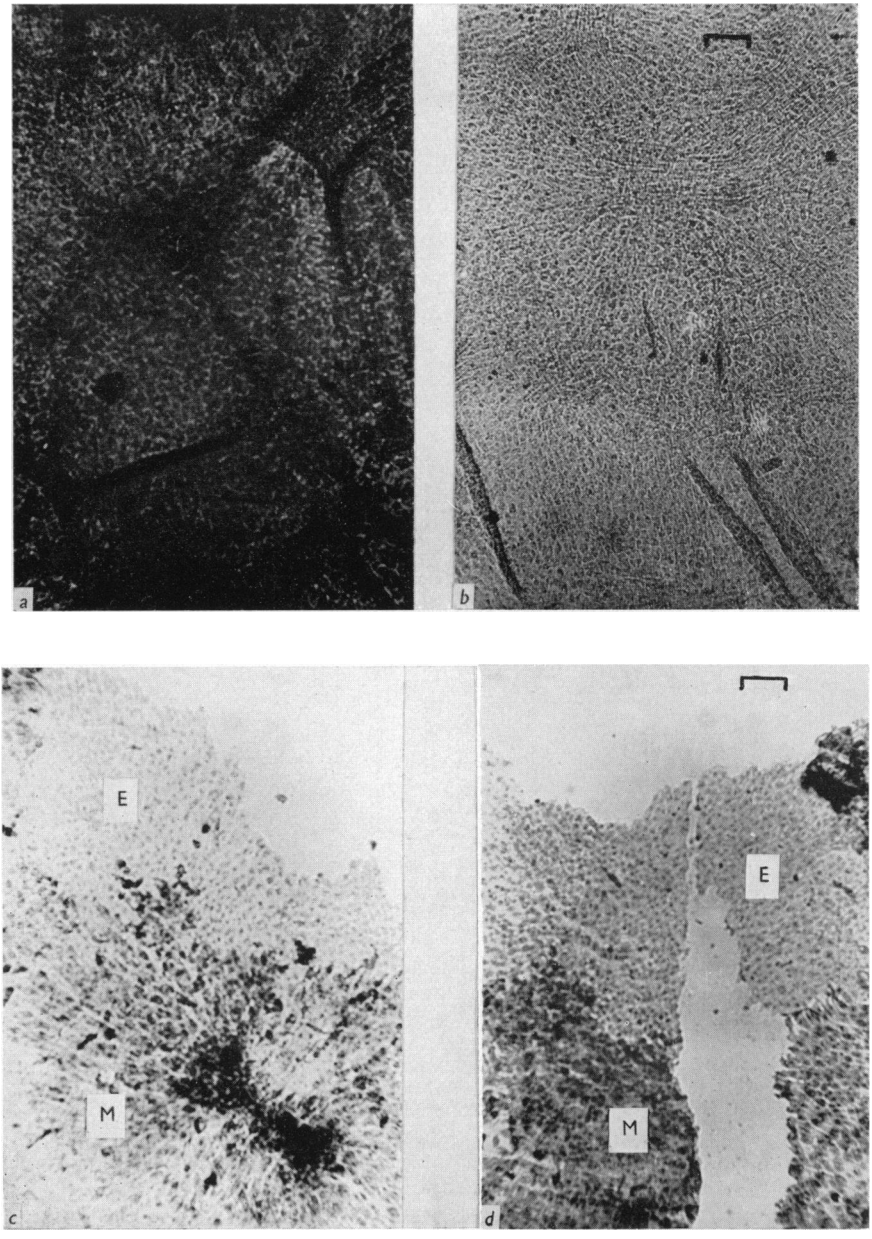
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EXPLANATION OF PLATES

PLATE 1

a–d. Staining for cholinesterase (Koelle method) in 9-day amniotic membranes. Acetylthiocholine and butyrylthiocholine used as substrates. Incubated for 6 hr at 37° C. Incubation solutions for control slides contained ($3{\cdot}6 \times 10^{-5}$ M) eserine. A well developed 'cross' figure is seen in fig. *c*, whereas fig. *a* shows what appears to be a developing 'cross' figure complex.

e. A 6-day chick amnion incubated with butyrylthiocholine for 4 hr at 37°C.

PLATE 2

a–b. Staining for esterase in two 9-day membranes, with α-naphthyl acetate as substrate. The membrane in *b* was incubated in 10^{-2} M DFP in Hanks's balanced salt solution for 60 min before staining. Both membranes were then incubated with the substrate for 90 min at 37° C. Calibration in *b* and *d* = 100 μ.

c, d. 8-day amniotic membranes stained for 100 min at 37° C (α-naphthyl acetate as substrate) to show darkly stained muscle layer and relatively unstained endothelial layer. E = endothelial layer, M = smooth-muscle layer.