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THE OCCURRENCE OF HOMOLOGUES OF ACETYLCHOLINE IN OX SPLEEN

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Although nearly twenty years have passed since Chang & Gaddum (1933) first drew attention to the lack of specificity of any one method for the bioassay of acetylcholine in tissue extracts and perfusates, little serious attention seems to have been given to the question as to whether cholinergic functions are invariably mediated by this substance.

The occurrence in animal tissues of other choline esters might be inferred from the recognition therein of two cholinesterases (Alles & Hawes, 1940; Mendel & Rudney, 1943) called by the latter authors 'true' and 'pseudo'. In a recent discussion of the physiological role of pseudo-cholinesterase (Whittaker, 1951), it was suggested that the enzyme, which hydrolyses propionyl and butyrylcholine more rapidly than acetylcholine, might function *in vivo* for the hydrolysis of choline esters other than acetylcholine. It was therefore decided to investigate the occurrence of choline esters in ox spleen. Spleen was chosen because of its high 'acetylcholine' activity (Chang & Gaddum, 1933) and because it is one of the few mammalian tissues from which acetylcholine has been isolated in sufficient quantity for identification chemically (Dale & Dudley, 1929). Realizing that the amounts of the choline esters present in tissues would be very small, paper chromatography was chosen as suitable for the separation (Whittaker & Wijesundera, 1952).

When dealing with spleen extracts the limitations of paper chromatography became evident, hence the direct use of extracts was abandoned and the technique applied to a concentrate of the tissue bases obtained by precipitation with reinecke salt (Kapfhammer & Bischoff, 1930). Active fractions were separately re-chromatographed until homogeneous components were obtained. The positions of the esters on the chromatograms were identified chemically, and after extraction the esters were characterized and estimated by biological assay.

In this way it has been possible to show that spleen contains, in addition to acetylcholine, two other choline esters, one identified as propionylcholine.

A brief report of this work has already appeared (Banister, Whittaker & Wijesundera, 1951).

METHODS

Materials and techniques

Choline esters. Synthetic choline esters were used, when possible, in the form of the perchlorate salts; as these are, in contrast to the chlorides, non-deliquescent and more stable. Acetyl-, propionyl- and valerylcholine perchlorates were prepared from the acid chlorides and choline perchlorate (Bell & Carr, 1947). Lactylcholine chloride was prepared by the method of Horenstein & Pählicke (1938). Propionylcholine chloride (PrChCl) was prepared with difficulty from propionyl-chloride and choline chloride; a better method (see Fourneau & Page, 1914) was to allow 2-chloroethyl propionate and trimethylamine to react at room temperature in dry benzene in a pressure flask. The propionylcholine chloride was then quickly collected by filtration, washed with a little dry benzene and stored in a vacuum desiccator containing paraffin wax shavings and conc. H_2SO_4. Acetylcholine chloride (AChCl) and other choline ester chlorides were obtained commercially.

Cholinesterase preparation. Horse serum cholinesterase was obtained from commercial horse serum by the method of Strelitz (1944). The preparation corresponded to her stage 3. It was stored at 0° C with chloroform as a preservative; 1 ml. of undiluted preparation contained 33,000 units of activity (1 unit$\equiv$1 μl. CO_2/hr) when incubated at pH 7·4 and 38° C with acetylcholine (30 mM) in a bicarbonate/carbon dioxide buffer.

Cholinesterase activity of spleen homogenates was studied manometrically using the Warburg technique (Ammon, 1933).

Paper chromatography was carried out as described by Whittaker & Wijesundera (1952). For large-scale runs solutions were applied as rectangles 2 × 23 cm with the long side at right angles to the direction of solvent flow. Strips, 3·5 cm wide, were cut from the chromatograms for assay. For transferring material from one chromatogram to another, elution with methanol was used. 90–100% of the acetylcholine was recovered when 10–100 μg quantities were eluted from 3·5 × 19 cm strips of paper with 25 ml. portions of methanol. The iodine vapour method of Brante (1949) proved useful for visualizing spots since the colour faded on removing the paper from the iodine and the material on the spot could then be eluted and assayed biologically.

The R_F values of synthetic esters were found to show considerable variation unless the conditions were rigorously controlled. Factors influencing the R_F value are: direction of solvent flow, ambient temperature, length of run, initial size of spot, presence of inorganic salts and other impurities. When possible, synthetic esters were run simultaneously as controls, but this was avoided in preparative chromatograms to eliminate the possibility of cross-contamination.

Estimation of choline esters

Chemical estimation of choline esters was effected by Hestrin's (1949) method using a Hilger absorptiometer 'Spekker' or a Summerson-Klett photometer.

Biological assays. (1) Frog rectus abdominis muscle: the method was essentially that of Chang & Gaddum (1933). The precautions mentioned by Feldberg & Hebb (1947) were taken to avoid any errors due to the presence of tissue bases or other substances in the extracts which might potentiate, simulate or inhibit the response of the muscle to acetylcholine. (2) Guinea-pig ileum: about 2 cm of ileum was suspended in a bath of Tyrode solution. The test solutions were added in small volumes and when a maximum contraction had been achieved the organ was washed out three times with prewarmed Tyrode before adding the next dose. (3) Leech muscle: assays were made with the eserinized and non-eserinized longitudinal muscle of the leech (*Hirudo medicinalis*) as described by MacIntosh & Perry (1950).

Biological and chromatographic characterization of choline esters

Chang & Gaddum (1933) demonstrated that biological methods might be used for the characterization of choline esters. The data collected in Table 1 show that the activities of a number of choline esters, relative to acetylcholine, differ sufficiently widely, when measured on various tissues, to permit characterization. For instance, propionyl- and butyrylcholine are more active than

TABLE 1. Relative potencies (acetylcholine = 100) and R_F values of choline esters

	Fall in blood pressure, uneserinized animals			Intestine				R_F*	
Ester of choline	(*c*)	(*d*)	(*r*)	(*g*)	(*r*)	Eserinized frog rectus	Eserinized leech	(*a*)	(*b*)
Acetyl	100	100	100	100	100	100	100	0·14	0·17
Propionyl	20[8]	0·5[9]	4[1] 1[2]	10[4] 2–10[5]	3[1] 30[3]	450[1] 150–200[5]	45[1]	0·22	0·27
n-Butyryl	0·8[8]	—	0[1]	—	0·24[1] 4[3]	115[1]	90[1]	0·28	0·29
n-Valeryl	—	—	—	—	0·2[1]	30[1]	0·9[1]	—	0·55
Palmityl	—	—	0·1–2[2]	0·1[6]	—	—	—	—	—
Glycollyl	—	—	0·25[1]	0·2[6]	0·22[1]	1[1]	0·13[1]	—	—
Lactyl	0·1[7]	—	—	0·1[6]	—	2[5]	—	0·18	—
Pyruvyl	—	—	10[1]	—	14[1] 5–10[3] 23[6]	13[1]	16[1]	—	—
Acetyl-β-methyl	100[8]	100–1000[9]	—	—	—	—	—	0·19	0·23
Benzoyl	—	0·01[9]	0·1[2]	0·025[10]	0·2[3] 0·1[10]	0·5–2·0[10]	—	0·28	0·30

* Ascending; (*a*) *n*-butanol saturated with water, (*b*) *n*-butanol–*n*-propanol–water (4 : 2 : 1). (*c*) cat, (*d*) dog, (*g*) guinea-pig, (*r*) rabbit.

[1] Chang & Gaddum (1933). [2] Hunt & Taveau (1911). [3] LeHeux (1921). [4] Wertheimer & Paffrath (1925). [5] Present work. [6] Abderhalden, Paffrath & Sickel (1925). [7] Dale (1914). [8] Simonart (1932). [9] Kahane & Lévy (1935). [10] Akcasu, Sinha & West (1952).

acetylcholine on frog and leech muscle and less active on mammalian smooth muscle, whereas acetyl-β-methylcholine is more active than acetylcholine on smooth muscle. Table 1 also includes the R_F values for *n*-butanol–water of the chlorides of the esters listed. It will be seen that acetylcholine has the lowest R_F value; each CH_2 group increases the R_F by about 0·07, isomers (cf. propionylcholine, acetyl-β-methylcholine) having almost identical values. The increments in R_F show that adequate separation and characterization of adjacent esters may be achieved by chromatography.

To test the possibility of using the technique of biological assay to localize and characterize choline esters in chromatograms, different combinations of choline esters were applied as a mixture to a strip of filter-paper. The several components of the mixture were applied alongside and run as controls. After irrigation and evaporation of solvent, the chromatograms were developed in iodine vapour. After tracing the position of the spots, the chromatograms of the mixture of esters were cut into numbered areas. Each area was cut up and extracted with 2 ml. portions of frog Ringer solution (pH 4) for 30 min at room temperature. The extracts were then decanted, stored if necessary at 0° C, and the choline ester content estimated by both the guinea-pig ileum and frog rectus methods, using as standards the same solutions of the esters as were used for the chromatograms.

Fig. 1 illustrates an experiment in which mixtures of acetylcholine and propionylcholine were applied to the paper in amounts of 10 μg each. Fig. 1*a* is a tracing of the chromatogram after development with iodine vapour. The amounts of ester recovered from each area are shown in

Fig. 1*b*. The method used for computing the quantities of acetylcholine and propionylcholine from the results of the differential assay is given in full in the Appendix. Fig. 1*b* illustrates that there is good recovery of both substances and that each is localized in its appropriate area. In the eluates of areas 3 and 4, identical results were obtained with both assay methods when acetylcholine was used as a standard, showing that the material in these areas (amounting to 87% of the recovered acetylcholine) was pure acetylcholine uncontaminated with propionylcholine. Similarly, areas 6 to 9 contained 98% of the recovered propionylcholine uncontaminated with acetylcholine. With 1 μg amounts of the esters good localization and separation were again

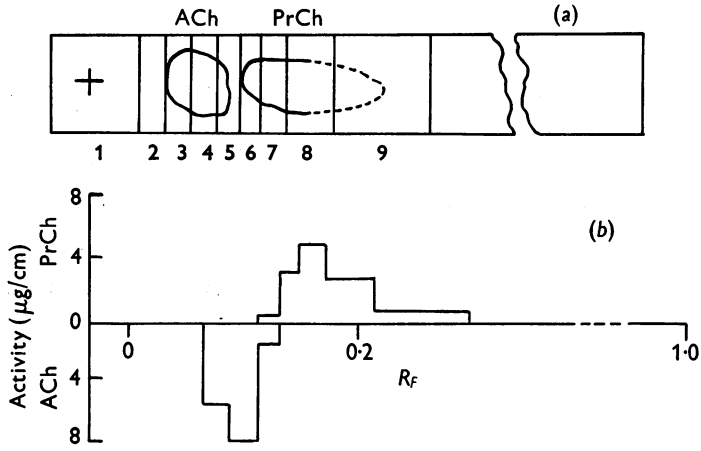

Fig. 1. Differential assay of chromatograms, run in *n*-butanol–water, of mixtures of acetyl- (ACh) and propionylcholine (PrCh). (*a*) Tracing of chromatogram of 10 μg of each ester. (*b*) Differential assay of chromatogram (*a*). The length of run was 23 cm. Total propionylcholine recovery, 80%. Total acetylcholine recovery, 75%.

achieved but the recovery was low. A correlation between localization by bioassay and by iodine vapour was always found: thus if diffuse spots, 'tailing' and overlapping were found, as occasionally happened, on developing chromatograms in which insufficient attention had been given to the technical details of the run, poor localization and overlapping were also found in the bioassays.

The calculation of the distribution of acetyl- and propionylcholine from the results of the differential assay assumes that the biological effect of the two esters is additive, i.e. that no potentiation occurs. This was found to be true for the guinea-pig ileum preparation, but a small degree of potentiation was occasionally found in the frog rectus assay. It is possible, therefore, in the eluates of areas containing both acetyl- and propionylcholine, that the calculated amount of the latter substance was too high. However, the area of overlap was small, and the results for the main portion of each component are unaffected.

Biological equivalence of chlorides and perchlorates

Difficulty was caused at one point in the work by the discovery that equimolar amounts of our samples of synthetic propionylcholine chloride and perchlorate did not give equal biological responses. This was traced to the greater instability of the chloride in the solid state. A fresh preparation of the chloride was compared with the perchlorate using the Hestrin (1949) colorimetric estimation for carboxylic esters. Equimolar solutions were found to give equal colour intensities and equal biological responses. The same was shown for acetylcholine. It is thus legitimate to use the more stable perchlorates as standards in the bioassays and this was the usual practice throughout the work. As the spleen preparations contained the bases as chlorides, the results of the assays are given in terms of the chloride.

Extraction of the esters from tissue

The alcohol extraction method of Dale & Dudley (1929) formed the first stage of our procedure. It was felt that extraction by aqueous trichloroacetic acid, while possibly being more efficient, would be less suitable as a preliminary to chromatographic separation, as the aqueous solution would contain more salt and would take longer to evaporate to a small volume. We first attempted to chromatograph aqueous extracts of the residue obtained when an alcoholic extract of spleen tissue was evaporated *in vacuo*. These extracts contained too much fat and inorganic salt to permit satisfactory chromatography. Accordingly, the tissue bases were purified further as the reineckates (Kapfhammer & Bischoff, 1930). Four large-scale preparations were carried out; a typical preparation is described.

Ten ox spleens (8·5 kg) were collected from the slaughterhouse, packed in ice-chips. After the outer sheath and fat had been removed as completely as possible, the spleens were cut into strips and passed through a mincer into ethanol (15 l.). The cutting and mincing were carried out in the presence of ethanol as recommended by Dale & Dudley (1929). The slurry of minced tissue and ethanol was brought to pH 4·5 with hydrochloric acid and left overnight in the cold-room with occasional stirring.

Next day the alcoholic extract was filtered off and evaporated (bath temperature 35–40° C) under reduced pressure to 800 ml. which was then made up to 5% with trichloroacetic acid, extracted repeatedly with ether, and evaporated to 25 ml. The addition of trichloroacetic acid was necessary to ensure complete extraction by the ether of the fat present and to avoid the formation of a stiff emulsion of the ether in the fat-laden solution.

The aqueous layer was treated with reinecke salt (3 g) dissolved in the minimum amount of water at pH 4·5. A voluminous pink precipitate formed and was collected by filtration after standing at 0° C for some hours. It was washed with a minimal quantity of ice-cold water and stored in a vacuum desiccator. Addition of more reineckate to the supernatant gave no further precipitate.

The reineckate precipitate (3·8 g) was dissolved in 200 ml. 50% (v/v) aqueous acetone and saturated aqueous silver sulphate (260 ml.) added until excess Ag^+ was present (*p*-dimethylaminobenzalrhodanine as external indicator). After silver reineckate had been removed by centrifuging, the solution was freed from SO_4^{2-} and Ag^+ by the addition of 10% (w/v) aqueous $BaCl_2$ until a slight excess of Ba^{2+} was present (rhodizonic acid as external indicator). After removal of $BaSO_4$ by centrifuging, the solution was reduced in volume to 10 ml. A trace of Ba^{2+} (which it was feared might interfere with the bioassays) was removed by adding dilute aqueous Na_2SO_4 until the solution ceased to give a Ba^{2+} reaction with rhodizonic acid. The total choline ester content of the preparation at this stage, assayed as acetylcholine chloride by the frog rectus, was 2·1 mg or 0·24 μg/g spleen tissue. This figure is much lower than that reported by Chang & Gaddum (1933), but there was evidence that the purification procedure entailed considerable losses of activity.

RESULTS

Paper chromatography of concentrate of tissue bases

In preliminary small-scale experiments, concentrates of tissue bases, prepared as described in Methods, were applied as spots to filter-paper and run in *n*-butanol. It was found on assaying a sample strip of the chromatogram by the frog rectus method, that acetylcholine activity was not confined to the area expected from the known R_F of acetylcholine but was also present in two other areas of higher R_F. Assay with the guinea-pig ileum showed comparable activity only in the area of lowest R_F. The three components giving rise to

these activities will be referred to as the S ('slow'), M ('middle') and F ('fast') components. From the R_F values and physiological behaviour of the S and M components, they were tentatively identified as acetyl- and propionylcholine, and attention was then directed to characterizing them further. The appropriate

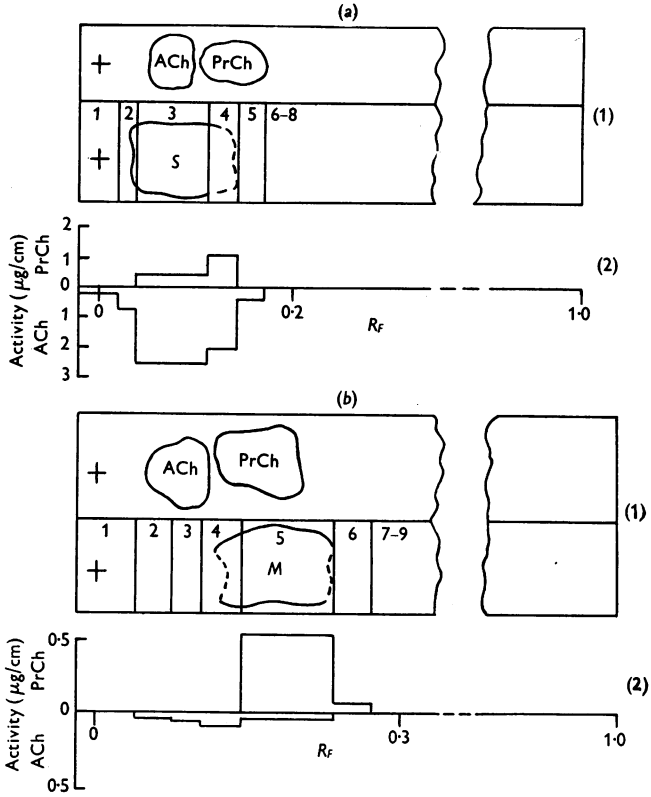

Fig. 2. Differential assay of (a) S component and (b) M component. The components were separated by descending chromatograms in n-butanol–water. a^1: Tracings of chromatogram of a mixture of synthetic acetyl- and propionylcholine, and S component. Length of run 32 cm. a^2: Results of differential assay of chromatogram of S component. b^1: Tracing of chromatogram of synthetic acetyl- and propionylcholine, and M component. Length of run 33·5 cm. b^2: Results of differential assay of chromatogram of M component.

areas were excised, extracted with methanol and re-chromatographed. Figs. $2a^1$ and b^1 are, respectively, the tracings of chromatograms of the S and M components and mixtures of synthetic acetyl- and propionylcholine run as controls. Figs. $2a^2$ and b^2 give the results of the differential assay of chromatograms of the S and M components in which the amounts of acetyl- and propionylcholine, which could have accounted for the responses of the two organs, have been calculated. It will be seen that the main component of S

behaved chromatographically and physiologically like acetylcholine, and the main component of *M* behaved like propionylcholine.

Each main component was, however, contaminated by the other and the conditions did not permit a sharp separation. The amount of material available did not allow further characterization, so a large-scale separation was carried out.

A concentrate of tissue bases from 8·5 kg of spleen tissue (prepared as described earlier) was submitted to large-scale chromatography by distributing the material equally on to twelve large sheets (27 × 50 cm) of filter-paper. By

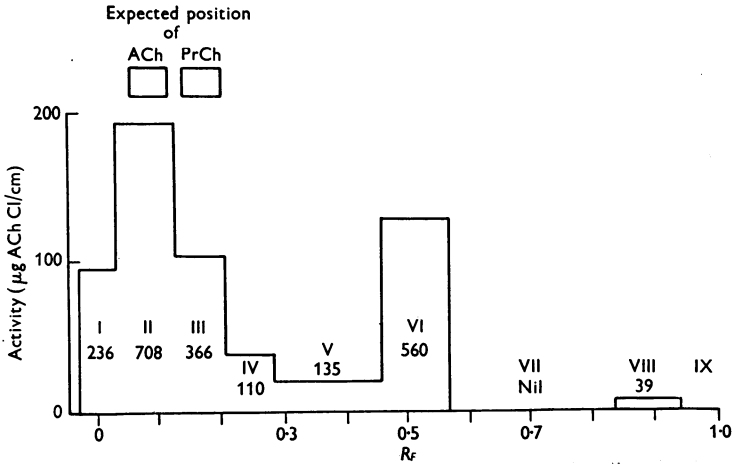

Fig. 3. Distribution of activity of concentrate of spleen bases along chromatogram. The total activity (as μg acetylcholine) and the area number are given for each assay. The length of run was 42 cm. Solvent *n*-butanol–water (descending).

drying the papers between applications it was possible to confine the material to a rectangle 2 cm in width, 2 cm from either edge and 12 cm from the bottom edge of the paper. After running, a control strip 3·5 cm wide from one of the sheets was taken for assay. From this the total activity in all sheets was calculated as μg/'acetylcholine'/cm of chromatogram. Its distribution along the chromatogram is given in Fig. 3. The total recovered activity amounted to 105% of that applied. In this large-scale run, the separation of the activity into discrete components was not as sharp as in previous experiments, but it was thought that the peak in area II (R_F 0·04–0·12) would correspond to acetylcholine, that the *M* component would be mainly in area III (R_F 0·12–0·21) and *F* component mainly in area VI (R_F 0·46–0·57). Accordingly, these areas were excised, extracted with methanol, the extracts from corresponding areas pooled and, after evaporation of the methanol, re-chromatographed.

Identification of acetylcholine in area II (Fig. 3): S component

Approximately 60% of the total activity of the chromatogram of the material from area II was recovered below R_F 0·14. This was re-chromatographed. The main component was identified chromatographically as acetylcholine (Fig. 4).

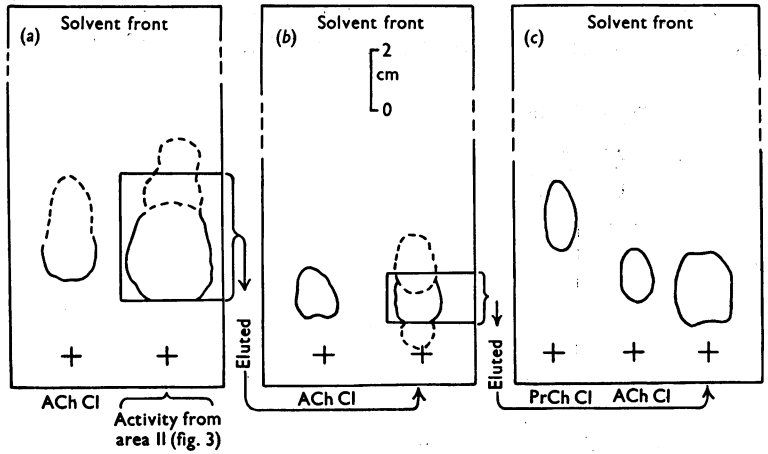

Fig. 4. Tracings of chromatograms of *S* component eluted from area II of Fig. 3. Re-chromatography of the compound: (*a*) descent in *n*-butanol–water, run 36 cm; (*b*) ascent in *n*-butanol–water, run 17·3 cm; (*c*) ascent in *n*-butanol–*n*-propanol–water (4 : 2 : 1), run 16 cm, showed that its behaviour was similar to acetylcholine chloride.

Chromatography of active material from area III (Fig. 3): M component

Approximately 65% of the total activity of the chromatogram of the material from areas III and IV was extracted between R_F's 0·13 and 0·29 and re-chromatographed. Fig. 5*a* is a tracing of one of three chromatograms. Only one component was present in amounts sufficient to give a colour reaction, though the crescent shape of the lower margin of the spot suggested that a second component might be following it. Fig. 5*b* gives the differential assay of this chromatogram. It will be noted that there is very little response of the guinea-pig ileum to the extracts of any portion of the chromatogram and that the peak of this response does not coincide with the peak of the frog rectus response. When the amounts of acetyl- and propionylcholine required to elicit the observed responses are calculated, as described in the Appendix, the results plotted in Fig. 5*c* are obtained. It will be seen that the material in section 5 of Fig. 5*a* (R_F 0·23–0·4) and corresponding to the spot, assayed as almost pure propionylcholine. The small ileum response was slightly larger than could be accounted for by the propionylcholine content of the area, but the observed

ratio of activities would have been obtained if the propionylcholine were contaminated with as little as 0·5% acetylcholine. The yield was 40 μg, corresponding to 22% of the acetylcholine activity found in area III of Fig. 3. The active substance of section 2 (Fig. 5*a*) corresponds to almost pure acetylcholine; here again, the ratio of activities in the two assays was not quite unity when acetylcholine was used as a standard, but the presence of 6% propionylcholine in the area would account for the observed ratio.

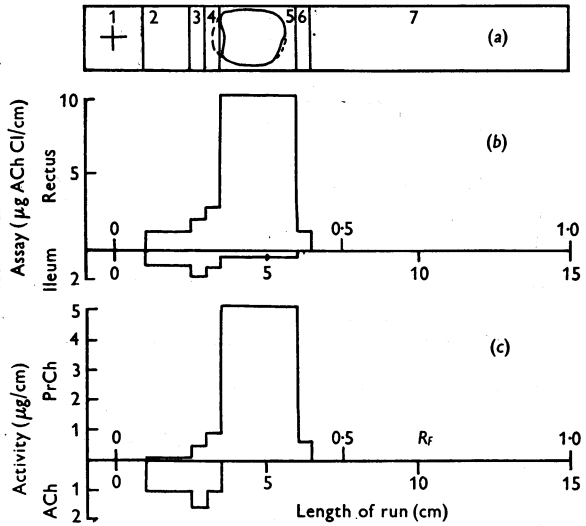

Fig. 5. *M* component. (*a*) Chromatogram of material from areas III and IV, Fig. 3. (*b*) Differential assay of chromatogram; results of assay. (*c*) Distribution of acetyl- and propionylcholine in chromatogram calculated from (*b*).

The component from section 5 (Fig. 5*a*) was eluted with methanol and rechromatographed (*a*) in aqueous butanol, (*b*) in *n*-butanol–*n*-propanol–water (4 : 2 : 1). Synthetic acetyl- and propionylcholine chlorides were run alongside as controls, thus permitting a direct comparison of the R_F of the *M* component with the R_F's of these esters. *n*-Propanol, in varying combinations, is the only solvent of a large number investigated which gives a separation as good or better than that achieved by *n*-butanol (Whittaker & Wijesundera, 1952). Tracings of the chromatograms are shown in Fig. 6. It will be seen that in each case, the R_F of the *M* component is the same as that of propionylcholine and distinctly greater than that of acetylcholine.

The *M* component was also shown in experiments with the frog rectus to be stable when boiled in acid solution and to be rapidly destroyed when boiled with alkali, thus behaving in these classical tests like a choline ester. Furthermore, choline was detected in a chromatogram of a sample of the substance

which had become inactive during storage. The *M* component was also destroyed by incubation with purified horse serum cholinesterase. This shows that it is not acetyl-β-methylcholine, which has a closely similar R_F value in all solvents so far tested, a conclusion already implicit in its behaviour in the bioassays.

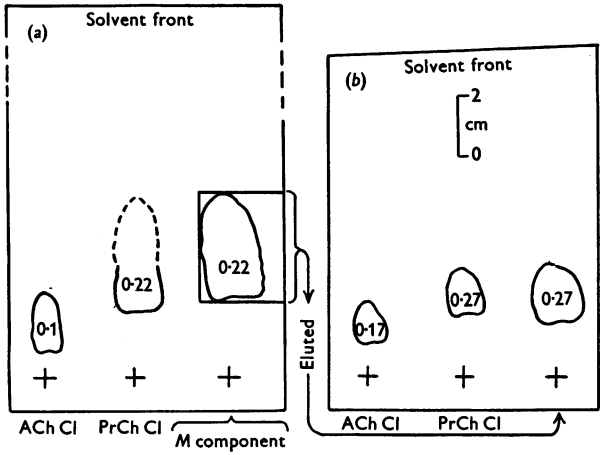

Fig. 6. Chromatograms of *M* component eluted from section 5 (Fig. 5*a*), compared with acetyl- and propionylcholine chloride. The figures within the spots are the mean R_F's. Ascending chromatograms (*a*) in *n*-butanol–water, run 18 cm; (*b*) in *n*-butanol–*n*-propanol–water (4 : 2 : 1), run 11 cm.

Chromatography of areas V and VI (Fig. 3): F component

The material in areas V and VI (Fig. 3) was eluted with methanol and chromatographed. From this chromatogram the active substance above R_F 0·3 was eluted and chromatographed again and assayed differentially. It will be seen (Fig. 7) that the peak of the *F* component lies between R_F's 0·6 and 0·7 (ascending chromatogram) which is distinctly higher than that of valerylcholine (mean R_F 0·55) the highest R_F of any synthetic ester available. That *F* is a choline ester was shown by the destruction of its activity (frog rectus) by M/3 alkali and by horse serum cholinesterase.

It was thought that if the *F* component was a choline ester it might be possible to detect choline chromatographically after alkaline hydrolysis. The R_F of choline is 0·03–0·05 R_F unit lower than that of acetylcholine. Accordingly, an amount of *F* component equivalent to 20 μg acetylcholine in 1 ml. was destroyed by heating in a boiling-water bath for 15 min with a drop of N-NaOH, the solution neutralized with N-HCl, evaporated to dryness *in vacuo* and extracted with methanol. The methanol extract was run as a single chromatogram. Acetylcholine chloride (20 μg) and choline chloride (10 μg) were similarly treated and run as controls. Fig. 8 is a tracing of the chromatogram

obtained. It will be seen that choline (R_F 0·09) is present in the hydrolysate of F; a complex pattern of fainter spots was also obtained. This suggests either that the F component is not a single entity or that it was still contaminated with other substances.

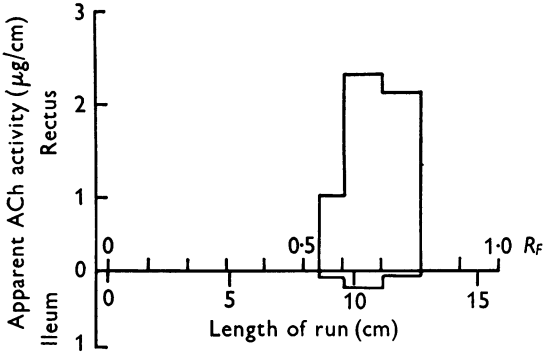

Fig. 7. Differential assay of F component from areas V and VI, Fig. 3. Material run as six ascending chromatograms in n-butanol–n-propanol–water (4 : 2 : 1). Total recovered activity was equivalent to 46 μg acetylcholine.

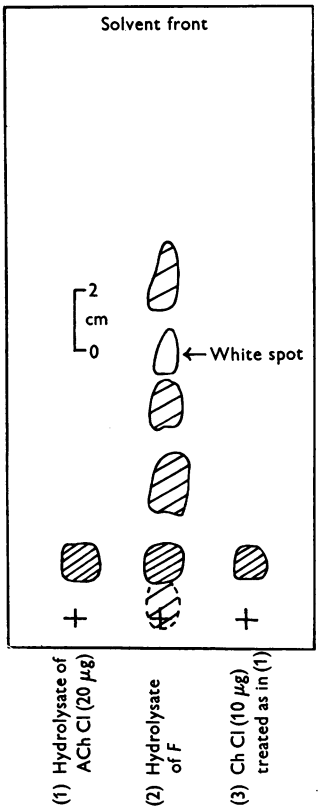

Fig. 8. Ascending chromatogram of alkaline hydrolysate of F. The shading indicates the relative intensity of the spots. R_F of choline chloride spot, 0·09. Solvent, n-butanol–water.

The possibility was considered that F was a choline ester of one of the keto-acids of importance in metabolism. Accordingly, we studied the absorption spectra of portions of hydrolysate to which had been added 4-nitro- and 2 : 4-dinitro-phenylhydrazine under the conditions used by Friedmann & Haugen (1943) in their colorimetric determination of keto-acids. Coloured complexes were formed with absorption maxima at 410 and 445 μm respectively, but the absorption curves were not identical with those of pyruvic, α-ketoglutaric,

acetoacetic acid or oxaloacetic acid. The R_F's of the first three of these acids are all below the unidentified spots in Fig. 8.

Inhibition of their activity by atropine and potentiation by eserine are generally considered to be characteristic of choline esters. Accordingly, these effects were studied on the frog rectus and leech muscle respectively.

The tracings in Fig. 9 show the depressant effect of 150 μg atropine on the response of the muscle (i) to 0·2 μg acetylcholine, (ii) to an amount of F equivalent to this amount of acetylcholine. In both cases the depression lasted for at least one contraction after the atropine had been washed out.

Fig. 10 shows the response of the longitudinal muscle of the leech to F and to acetylcholine before and after eserinization. The potentiating effect of eserine is marked in both cases. Both these experiments therefore support the conclusion that F is a choline ester.

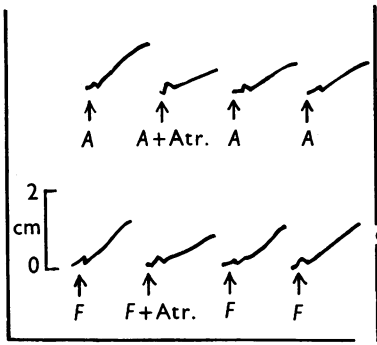

Fig. 9.

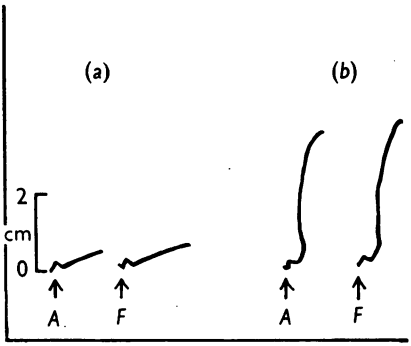

Fig. 10.

Fig. 9. Top line: inhibition of response of frog rectus to 0·2 μg acetylcholine (A) by 150 μg atropine (Atr.). Bottom line: similar inhibition of the response to F (fast component). Test solutions were washed out after 90 sec.

Fig. 10. Contractions of longitudinal muscle of leech. A: 0·03 μg acetylcholine, F: fast component, (*a*) before eserine, (*b*) after sensitization with 1/100,000 eserine. Test solutions were washed out after 90 sec.

Cholinesterase activity of the spleen

Homogenates of ox spleen possess a fairly high cholinesterase activity. Table 2 gives the rates of hydrolysis of several esters expressed as μl. CO_2 liberated/hr/g wet weight of tissue at 38° C. It will be seen that the specificity pattern of the whole homogenate corresponds to neither of the classical

TABLE 2. 'Specificity' of cholinesterase of whole spleen homogenate

Ester	Rate of hydrolysis (μl. CO_2/g tissue/hr) at concentrations indicated below: 30 mM	6 mM
Acetylcholine	536	624
Propionylcholine	512	584
Butyrylcholine	664	—
Acetyl-β-methylcholine	456	—
Benzoylcholine	5	—

cholinesterase types nor to a mixture of them. Thus in spite of the failure to hydrolyse benzoylcholine at an appreciable rate, butyrylcholine, a substrate which is hardly attacked by 'true' cholinesterases, is split faster than acetyl- or propionylcholine. No attempt was made to decide whether the overall specificity pattern of the spleen homogenate was that of a single cholinesterase or was the composite pattern of a mixture of enzymes.

DISCUSSION

Tissues from which acetylcholine has been isolated in chemically identifiable quantities are spleen (Dale & Dudley, 1929), brain (Stedman & Stedman, 1937), blood (Kapfhammer & Bischoff, 1930; Dudley, 1933), intestine of the horse (Chang & Gaddum, 1933), and the nervous system of *Octopus vulgaris* (Bacq & Mazza, 1935). It has also been physiologically characterized in extracts of human placenta (Chang & Gaddum, 1933), and in the perfusate of the superior cervical ganglion (Feldberg & Gaddum, 1934) and the stomach (Dale & Feldberg, 1934) during nervous stimulation.

The work described in the paper has revealed the presence in ox-spleen extracts of two choline esters in addition to acetylcholine. One of them has been identified physiologically and chromatographically as propionylcholine. The other ester, which we have referred to as the *F* component, remains unidentified and may not be a single substance. In their classical work on the isolation of acetylcholine from horse spleen, Dale & Dudley used the rabbit jejunum for assaying the activity of their fractions. As propionylcholine and the *F* ester are relatively inactive on the mammalian intestine, they might have been present in the fractions which Dale & Dudley discarded.

Apart from the phospholipids and their breakdown products the only other choline derivatives which, so far as we are aware, have been reported to occur in animal tissues, or indeed in biological material generally, are murexine (Erspamer & Dordoni, 1947), and a form of bound choline in the oxytocic hormone (Freudenberg & Biller, 1936). The constitution of neither of these derivatives is known. Propionylcholine is thus the first simple physiologically active derivative of choline of known constitution, other than acetylcholine, to be isolated from biological material.

In considering the possible identity of the *F* component it seems unlikely that it is the ester of a homologue of acetic acid. The R_F of *F* corresponds to an *n*-acylcholine, one or more CH_2 groups above valerylcholine. The physiological properties of these higher homologues are not known, but the trend (Table 1) is clearly towards low absolute activities and a high rectus/intestine activity ratio. The activity of *F* per unit weight is not known, but the impression was gained from the intensity of the spots that this could not be very different from that of acetylcholine, and the differential assay suggested an activity ratio of about 10.

It is possible that *F* is a choline ester of an amino-, hydroxy- or keto-acid. It is not lactylcholine, as this has too low an R_F value and has a very low biological activity. We have not had any amino-acid esters available for comparison, but those described in the literature, glycylcholine (Abderhalden *et al.* 1925), alanyl- and leucylcholine (Freudenberg & Keller, 1938) have been reported to have very feeble physiological activity. There is some evidence that *F* is a keto-acid ester, in that *F* and its alkaline hydrolysate reacted with 2 : 4-dinitrophenylhydrazine and 4-nitrophenylhydrazine to give substances with absorption spectra in the visible and near ultra-violet, but the presence of pyruvate, acetoacetate, α-ketoglutarate or oxaloacetate could not be confirmed. Unfortunately, physiological data are available for only one keto-ester, pyruvylcholine. It is interesting that this alone, of all choline esters of naturally occurring acids other than the immediate homologues of acetylcholine, is fairly active, and although its differential response precludes its being identical with *F*, it is possible that some other keto-acid might show the required properties.

A considerable proportion of the total 'acetylcholine' activity of ox spleen as assayed by the frog rectus must be due to propionylcholine and *F*. It is not possible to say with certainty what this proportion is, since considerable loss of activity occurred both during the initial separation of the tissue bases and the repeated chromatography of the various active fractions. If the activities of areas III and IV of Fig. 3 are taken to be mainly due to propionylcholine and those of areas V and VI to *F*, these substances account for about 20 and 30% respectively of the activity of the original extract.

Recent publications by Crawford (1951) and Smith (1952) illustrate some of the pitfalls in the isolation of biologically active materials from tissues. Crawford identified lactyladrenaline and lactyl-noradrenaline in extracts of an adrenal medullary tumour which also contained the free bases. If, however, the acid ethanol extract was not evaporated to dryness, no lactyl esters were detected, and he concluded they were artifacts. It is possible that propionylcholine might make its appearance in a similar way from choline, an ubiquitous tissue constituent, and propionic acid, formed with other lower fatty acids (cf. Gray, Pilgrim, Rodda & Weller, 1951) in the ruminant stomach and thence absorbed into the blood stream (Reid, 1950). We do not think this likely, as care was taken to avoid evaporating our extracts to dryness and the bath temperature was not allowed to exceed 40° C. Smith (1952) has pointed out that a single substance may, in the presence of salt and in an improperly equilibrated column, migrate as more than one band. In our work, any salt present would have been eliminated after the first chromatogram, and active fractions invariably ran as single components after elution and re-chromatography. There is evidence, however (cf. Abdon & Hammarskjöld, 1944) that the acetylcholine in tissues is present as an inactive complex from which it is liberated by acid extraction fluids. We have no information as to whether the

same applies to the other choline esters in our spleen extract and in this sense they may be artifacts.

The present work gives no clue as to the physiological role of propionylcholine and the *F* component in ox spleen, but indeed there is as yet no satisfactory explanation for the large amounts of acetylcholine found in this organ.

With regard to the metabolism of the choline esters in ox spleen, we have shown that this tissue, though almost without action on benzoylcholine, can hydrolyse propionyl- and butyrylcholine at a rate comparable to that of acetylcholine. Mendel & Rudney's statement (1944) that ruminant tissues contain no pseudo-cholinesterases was possibly based on an inability to hydrolyse benzoylcholine and may be misleading unless the limitations of specificity studies with only two substrates, acetyl-β-methylcholine and benzoylcholine, are borne in mind. No attempt has yet been made to study the synthesis of propionylcholine and *F* component but the observation of Korey, de Braganza & Nachmansohn (1951) that purified squid ganglion choline acetylase can synthesize propionylcholine from added propionic acid and choline suggests that essentially the same enzyme mechanisms may be involved as in acetylcholine synthesis. Propionate has generally been considered to be metabolically inert but propionate originally derived from gastric fermentation or from some other source might be utilized for the synthesis of propionylcholine *in vivo*. There is evidence that enzymes dependent on coenzyme A exist in animal tissues and bacteria which are capable of activating not only acetate but also other organic ions, e.g. benzoate (Chantrenne, 1951), succinate (Sanadi & Littlefield, 1951) and propionate (Stadtman, 1952), in acylation reactions. Such observations suggest a fruitful line of attack on this problem.

SUMMARY

1. A method has been worked out for separating and characterizing choline esters using a combination of paper chromatography and differential bioassay. The esters are characterized both by their R_F and their differential response when assayed by (*a*) the frog rectus, (*b*) the guinea-pig ileum.

2. The method was applied to extracts of the bases present in ox spleen, after preliminary concentration by reineckate precipitation.

3. In addition to acetylcholine, two other active esters were detected in the chromatograms. One of these was identified chromatographically and physiologically as propionylcholine. The other (R_F 0·65 in *n*-butanol–water) was shown to be a choline ester but has not yet been identified.

Most of this work was carried out in the Departments of Biochemistry and Physiology, University of Oxford, during the tenure by one of us (V. P. W.) of a Medical Research Council grant-in-aid. We are grateful to Dr P. W. Kent for the preparation of lactylcholine and to the University of Ceylon for a Travelling Fellowship (S. W.).

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APPENDIX

Method of estimating acetylcholine and propionylcholine by differential bioassay.

Let i = total quantity in area as μg acetylcholine giving an equivalent response as assayed by the ileum.

r = total activity in area as μg acetylcholine giving an equivalent response as assayed by the rectus.

P_i = μg propionylcholine equivalent to 1 μg acetylcholine in the ileum.

P_r = μg propionylcholine equivalent to 1 μg acetylcholine in rectus.

These amounts of acetyl- and propionylcholine (x, y) in the area are the solutions of the simultaneous equations

$$x + y/P_i = i,$$
$$x + y/P_r = r,$$

from which it is seen that

$$x = \frac{iP_i - rP_r}{P_i - P_r}; \quad y = \frac{(r-i)\,P_iP_r}{P_i - P_r}.$$