The Identification of Propionylcholine as a Constituent of Ox Spleen

By J. E. GARDINER and V. P. WHITTAKER
Department of Physiology, University of Cincinnati College of Medicine, Cincinnati, Ohio

(*Received* 3 *November* 1953)

Propionylcholine has recently been identified by Banister, Whittaker & Wijesundera (1953) as a constituent of ox spleen. The identification was made on the basis of R_F value and physiological properties. It seemed desirable to confirm the identification by an unequivocal physical method such as infrared spectroscopy. Paper chromatography, the method of separation used in the earlier work, is not well suited for isolating propionylcholine in the quantities required, and an improved method of separation has been devised which makes use of chromatography on ion-exchange resins.

Ox spleen was originally selected by Banister *et al.* on account of the high content of acetylcholine or acetylcholine-like substances reported for it by Dale & Dudley (1929) and Chang & Gaddum (1933). It has not been possible to confirm these findings either in the earlier work or in the present study. This discrepancy and a possible explanation for it is considered in the discussion.

The work described in this paper was communicated to the XIXth International Physiological Congress, Montreal (Gardiner & Whittaker, 1953).

EXPERIMENTAL

Materials and methods

Ion-exchange resins. Amberlite XE-97 (lot no. 5258, 100–500 mesh) was washed by sedimentation in tap water to remove fines, transferred to columns and subjected to either of the following treatments:

(1) The resin was washed with N-HCl using double the volume required to give a metal-free effluent (i.e. flame test negative), then with water until the pH of the effluent exceeded 5 and finally washed with 0·1 M-NaH_2PO_4 until the pH of the effluent was the same as that of the influent.

(2) The resin was: (*a*) washed with N-NaOH, using double the volume required to change the tint of the resin from off-white to white; (*b*) removed from the column, washed by sedimentation with three to four changes of water and repacked in the column; then (*c*) washed with N-HCl, using double the volume required to change back the colour of the resin. (*d*) Step (*b*) was repeated. (*e*) Steps (*a*)–(*d*) were repeated twice. The column was then washed with water until the pH of the effluent was greater than 5 and equilibrated with 0·02 M-NaH_2PO_4.

Batches of prepared resin were stored under 0·1 or 0·02 M-NaH_2PO_4 until needed.

The basic resin Amberlite XE-98 (lot no. 4801) was powdered in a mortar, washed to remove fines, transferred to a column and converted into the perchlorate form by washing with 0·1 N-$HClO_4$ until free from chloride.

Amberlite XE-59 was converted into the hydroxide form by washing with 5% (w/v) NH_3 until free from chloride. Ammonia was removed by washing with boiled-out water until the pH fell below 7·4.

Other materials. Reagents and solvents whenever possible were A.R. A solution of $AgClO_4$ was prepared by the repeated evaporation *in vacuo* of $AgNO_3$ and $HClO_4$ at 100°, followed by neutralization of excess acid with Ag_2CO_3 and filtration. The methanol used in the final stages was Eastman Kodak 'Spectro' grade. Choline esters were as described by Banister *et al.* (1953).

Bio-assay of choline esters was carried out on the frog rectus abdominis muscle (Chang & Gaddum, 1933) or on the isolated guinea pig ileum (for details see Banister *et al.* 1953). When crude extracts were being assayed, allowance was made for interfering substances by the method of Feldberg & Hebb (1947), but when large numbers of assays had to be made on the fractions from a chromatographic run, this precaution was omitted, and the flanking procedure abbreviated somewhat.

The separate estimation of acetyl- and propionyl-choline in a mixture, particularly if other tissue constituents are present in appreciable quantities, presents some difficulty, especially as the relative potencies of the two esters in a given test system vary somewhat from one preparation to another and are influenced in an unpredictable manner by the composition of the extract. Nevertheless, it was necessary to have some method of measuring the activity of the various fractions in order to assess the efficiency of the purification procedure. The unit of activity adopted was the amount of synthetic acetylcholine perchlorate in m-μmoles giving an equivalent response on the frog rectus abdominis muscle (or guinea pig ileum, if specifically stated) when tested in a concentration range over which the dose/response curve of the preparation was approximately linear. Owing to the difficulties mentioned above, yields and recoveries based on activities measured in this way must be regarded as giving approximate indications only.

Chromatography. The columns were run at room temperature (25–35°). The effluent was collected in fractions of constant volume by means of an automatic fraction collector.

Chromatographic separation of choline esters

Choline and its esters, like other quaternary nitrogen bases, would be expected to exchange with the replaceable cations of acidic resins. Two procedures for the separation of these bases on acidic resins were considered: adsorption on the hydrogen form of the resin followed by displacement chro-

matography with acid solutions or adsorption on to the resin in its metal salt form (e.g. sodium form) followed by partition chromatography with solutions of salts of the metal. The first of these methods was found to be impracticable. Resins with sufficient salt-splitting capacity (i.e. sulphonic acid resins) caused rapid destruction of the esters, presumably because of the low internal pH of the resin particles. The second method permits the use of buffer solutions, but has the disadvantage that the active material has to be separated from a large excess of buffer salt. The strong acid resins again proved unsatisfactory, even with buffers close to the pH of optimum stability of the esters. Good separation (with recoveries of 90–100%) was however obtained with the carboxylic acid resin Amberlite IRC-50 or its fine-grain modification XE-97 and phosphate or acetate buffers at pH 4·3–4·5, values as near the pH of maximum stability of the esters (about pH 4) as is compatible with retention by the resin of a useful amount of base-binding capacity.

Experiments with synthetic esters showed that acetylcholine comes off the resin first, followed by propionylcholine, then by butyrylcholine. Succinylmonocholine and acetyl-β-methylcholine (β-acetoxypropyltrimethylammonium chloride) have about the same affinity for the resin as acetylcholine, but succinyldicholine is very firmly bound and needs solutions of pH 1–2 for its elution (unpublished work by I. A. Michaelson). Choline comes off slightly ahead of acetylcholine. When the pH of the eluting buffer is raised, the affinity of the resin for acetyl- and propionyl-choline first increases, then decreases, being maximal at about 5·5–6·0, and the degree of separation is diminished. The pH of maximum affinity for choline is somewhat higher (about 7·0) so that at this pH acetyl- and propionyl-choline emerge from the column together, followed by choline. Preliminary treatment with alkali, as in procedure (2), reduces the affinity of the esters for the resin, perhaps by removing material of low molecular weight, but gives sharper separation when used with a dilute (0·02 M) buffer. The alkali-treated resin was therefore preferred for runs with partially purified fractions. The separation obtained with synthetic acetyl- and propionyl-choline can be seen in Fig. 4.

Isolation of propionylcholine from ox spleen

Concentration of spleen bases. This was carried out according to the scheme outlined in Fig. 1. Up to the stage of reineckate precipitation, the procedure was essentially as described by Banister *et al.* (1953). Chromatography at this stage was unsatisfactory, apparently because columns of any practical size were overloaded by the large amount of inactive electrolytes present. These were partly eliminated by precipitating the tissue bases as their reineckates; further quantities were removed by decomposing the reineckates with silver perchlorate and making

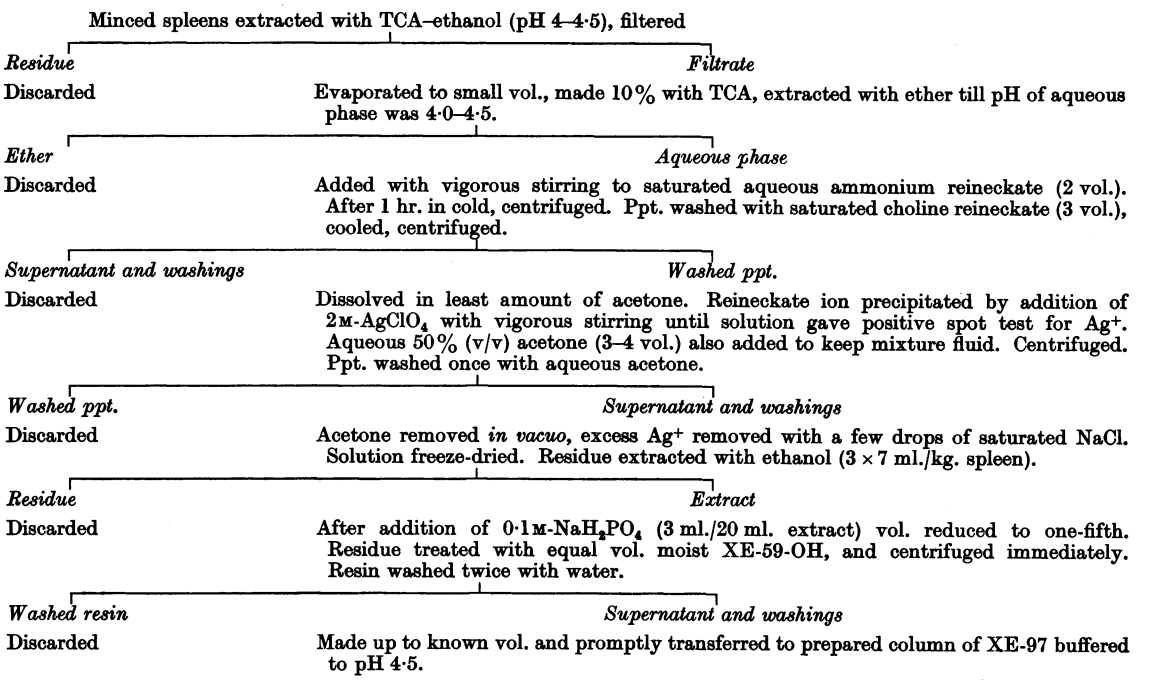

Fig. 1. Flow diagram showing the extraction of choline esters from ox spleen.

use of the sparing solubility of the inactive perchlorates (mainly creatine, potassium and choline) in ethanol.

A typical preparation was as follows. Six spleens (2·9 kg.) were removed from the animals 20–40 min. after death and immediately packed in chipped ice. Extraction was commenced in the laboratory cold room 1 hr. later. After removal of the capsule and adherent fat, the spleens were cut into strips and minced into ethanol (3 kg./kg. spleen). The cutting and mincing were carried out in the presence of chilled ethanol (cf. Dale & Dudley, 1929). After acidification with 50% (w/v) trichloroacetic acid (TCA), the mixture was left overnight in the cold room with stirring. After filtering through muslin and then through cellulose powder in a Büchner funnel, the filtrate was evaporated *in vacuo* at 37° and fat removed by extraction with ether. The aqueous residue (202 ml.) had a total activity of 4040 units. A considerable amount of activity was lost in the numerous assays at various stages, in the reineckate precipitation and the subsequent ethanol extractions (Fig. 1) leaving 1625 units (40%) for chromatography.

Chromatography of tissue choline esters. Fig. 2 shows the distribution of this active material in the effluent after chromatography on XE-97 prepared according to procedure (1). The initial peak of rectus-stimulating material was identified as choline on the basis of its position on the chromatogram, its predominantly rectus-stimulating activity (the choline : acetylcholine activity ratio for the frog rectus is 10 times that for the ileum), and its stability when boiled in alkaline solution. The next peak was identified as acetylcholine on the basis of its position on the chromatogram, the concordance of the rectus and ileum assays when synthetic acetylcholine was used as the standard, and alkali lability. The quantitative discrepancy between the rectus and ileum peaks is probably not significant, since some inactivation of the material may have taken place between the two series of assays, and there may have been substances present in some of the fractions which modified the response of the two organs to different extents. The third peak was identified as propionylcholine on the basis of its position immediately after acetylcholine on the chromatogram, its predominantly rectus-stimulating properties and its alkali lability. The activity recovered in the second and third peaks was 410 and 1040 units respectively, together comprising 91% of the alkali-labile activity before chromatography.

Histamine was identified as an ileum-stimulating substance whose alkali-stable activity was blocked by the antihistamine *N*-*p*-methoxybenzyl-*N'N'*-dimethyl-*N*-α-pyridylethylenediamine maleate (1 μg./ml.) (Merck and Co.) in fractions collected after 3·5–4·0 l. or in acid (0·1 N) eluates of columns from which choline esters had already emerged. Ninhydrin-positive material (basic amino acids) was present in fractions up to 200 ml. Butyrylcholine was not present in detectable amounts.

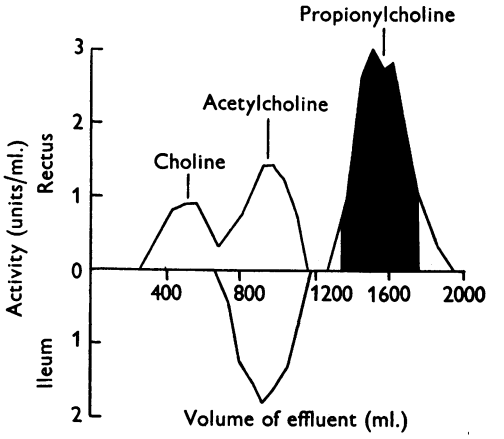

Fig. 2. Chromatography of concentrate of spleen tissue bases on XE-97. For definition of activity units see text. Column size, 27 × 2·4 cm.; eluting agent, 0·1 M-NaH_2PO_4; volume of fractions, 10 ml.; flow rate, 50 ml./hr.

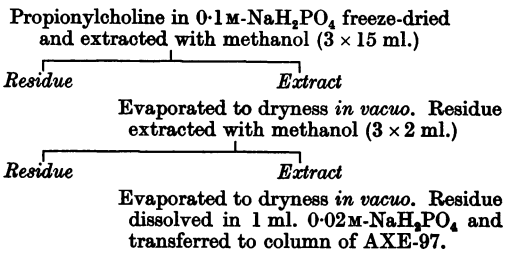

Fig. 3. Separation of propionylcholine from sodium dihydrogenphosphate.

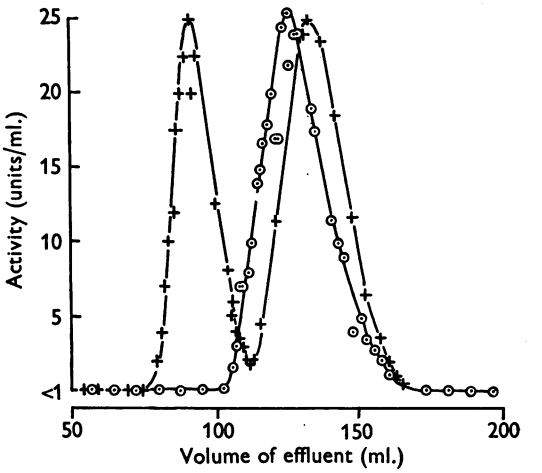

Fig. 4. Chromatography of spleen propionylcholine on AXE-97. Activity units as defined in text. Column size, 10·5 × 0·8 cm.; eluting agent, 0·02 M-NaH_2PO_4; volume of fractions, 1·2 ml.; flow rate, 5 ml./hr. Circles, spleen propionylcholine; crosses, calibration run with synthetic acetylcholine (0·4 μmoles) and propionylcholine (0·5 μmoles).

Fractions containing propionylcholine (blacked-in portion of Fig. 2, 910 units) were united with 2060 units of propionylcholine prepared in the same way from 5·4 kg. spleen. The combined material (2970 units in 1460 ml. 0·1 M-NaH_2PO_4) was divided into two portions, each of which was partially freed from sodium phosphate as shown in Fig. 3 and further purified by chromatography on AXE-97 using 0·02 M-NaH_2PO_4 as eluting agent. Fig. 4 (circles) shows the distribution of activity in the eluate from one of these runs. A calibration run (crosses) performed later with synthetic acetyl- and propionyl-choline shows that the active material from spleen runs like propionylcholine on the column. The slight rightward displacement of the control peak is accounted for by changes in rate of flow and ambient temperature. The total activity recovered after chromatography was 1650 units; this was 83 % of the activity placed on the columns, 56 % of the combined activity from the first chromatograms or 12 % of the ethanol-extractable activity of the original tissue.

Differential bio-assay of spleen propionylcholine. Although propionylcholine is relatively inactive on the ileum (2–10 % that of acetylcholine) it can be assayed on this preparation. Owing to the different relative potencies of different choline esters on the rectus and ileum (see Table 1, Banister *et al.* 1953) a comparison of the assay results obtained with the two types of muscle is one way of characterizing the esters (Chang & Gaddum, 1933). The assay results will, in general, only agree if the ester used as the standard is identical with the ester present in the 'unknown'. In Table 1 the results of two such differential assays are shown for the two samples of spleen propionylcholine prepared as described in the previous section. Using acetylcholine as standard, widely discordant results were obtained with the two assay systems, the 'unknown' appearing to have up to 60 times the activity when assayed with the rectus as with the ileum. When propionylcholine was used as a standard the two assays gave identical results.

Infrared spectrum of propionylcholine. Preliminary experiments with synthetic acetyl- and propionyl-choline perchlorates (kindly carried out by Dr A. R. H. Cole, Oxford University) had shown that the two esters could be distinguished on the basis of their infrared spectra. We had available 1650 units of spleen propionylcholine derived from a total of 8·3 kg. tissue and a further 210 units obtained in a pilot experiment from 1·7 kg. spleen; 1860 units in all, corresponding to about 300 μg. propionylcholine perchlorate. This precluded the use of a mulling technique and suggested the use of a spectrometer with reflecting microscope attachment (cf. Barer, Cole & Thompson, 1949). Dr Darwin L. Wood, of the University of Michigan, kindly undertook to place his microspectrometer at our disposal; his report is presented in the Appendix.

For spectroscopic examination it was necessary to separate the spleen propionylcholine from the buffer solution and mount it in a suitable manner. As spectroscopic identification of the naturally occurring ester isolated as the chloride by paper chromatography had been defeated, over 2 years ago, by the hygroscopic nature and instability of this salt, it was also considered essential to convert the ester into the more stable perchlorate. Accordingly, samples containing the ester (750 units in 50 ml.) were freeze-dried and the residue was extracted with methanol (2 × 10 ml.). After removal of the methanol, the residue was re-extracted with methanol (2 × 1 ml.); to this was added $CHCl_3$ (10 ml.). After 1 hr. in the cold, the mixture was centrifuged to remove sodium phosphate and the supernatant evaporated. The residue was transferred in a total volume of 5 ml. boiled-out water to an Amberlite XE-98-ClO_4 column (4 × 0·8 cm.) to convert the ester into the perchlorate. The effluent was evaporated and the ester transferred in a small volume of methanol to a 5 × 5 mm. silver chloride plate where it was deposited as a spot 2 mm. in diameter. The sample still contained a considerable amount of perchlorate derived from the NaH_2PO_4 of the eluting solution, but fortunately the inorganic ions present did not interfere with the identification of the biological material as propionylcholine.

Synthesis of propionylcholine by choline acetylase

The biological occurrence of propionylcholine raises the question of its synthesis. The most obvious route is via propionylcoenzyme A and the choline acetylase system, especially as enzymes are now known which convert the whole range of fatty acids, also succinic and benzoic acid, into acyl-coenzyme A derivatives. To test this point, the experiment summarized in Table 2 was performed. The source of choline acetylase was pigeon brain, already known as a rich source of cholinesterase (Whittaker, 1953). To identify the active component,

Table 1. *Differential bio-assay of spleen propionylcholine*

Sample	Choline ester used as standard	Bio-assay (m-μmoles/ml.) Rectus	Ileum	Ratio, rectus/ileum
I	Acetylcholine	2·75	0·1	27·5
	Propionylcholine	2·5	2·5	1·0
II	Acetylcholine	30	0·54	59
	Propionylcholine	30	32	1·07

Table 2. *Synthesis of choline esters by pigeon-brain choline acetylase*

An acetone powder of the brain was extracted with ice-cold 0·9% (w/v) NaCl for 1 hr. The supernatant after centrifuging (15 000 *g*) was treated for 0·5 hr. with 0·5 vol. XE-59-Cl in the cold (cf. Balfour & Hebb, 1952). The system contained in 2 ml., resin-treated extract from 40 mg. acetone powder, 50 μmoles cysteine, 100 μmoles KCl, 20 μmoles choline chloride, 1 μmole NaH_2PO_4, 25 μmoles NaF, 3·3 mg. adenosine triphosphate (Na_2 salt) and 60 μg. eserine (as sulphate); pH was adjusted to 7. Coenzyme A (Pabst Laboratories, Division of Pabst Brewery Co., Milwaukee, Wis.; 70% pure, 36 units) and Na acetate or propionate (20 μmoles) were added as indicated. The mixture was incubated for 1 hr. at 37°, brought to pH 4, boiled, centrifuged and the supernatant used for assays and chromatography. Activity expressed as μg. acetylcholine chloride/g. acetone powder.

	Activity after 1 hr.	Chromatographic identification of active material
Extract + CoA	145	Acetylcholine
Extract + acetate	225	Acetylcholine
Extract + CoA + acetate	3100	Acetylcholine
Extract + CoA + propionate	1100	Propionylcholine + trace of acetylcholine

extracts were freed from choline by chromatography on AXE-97 at pH 7·4; the active fractions were freeze-dried and run on AXE-97 at pH 4·5 to separate acetyl- and propionyl-choline. As seen in Table 2, propionate gave rise to propionylcholine in this system; the trace of acetylcholine probably arose from acetyl donors which, as shown by the first result recorded, were present in the preparation. Berman, Wilson & Nachmansohn (1953) have recently reported that purified squid-ganglion choline acetylase can synthesize propionylcholine from propionylcoenzyme A or an appropriate donor system; their product was not isolated, however.

DISCUSSION

Propionylcholine from ox spleen has now been identified as such by chromatography on paper and on columns of ion-exchange resin, by differential bio-assay and by infrared spectroscopy. The technique of chromatography on ion exchange resin has several advantages over paper chromatography (Whittaker & Wijesundera, 1952) for separating choline esters; the separation is sharper and less affected by impurities, and the recoveries are excellent. The chief disadvantages for our purposes were the difficulty of separating the esters from the buffer solution and the dilution of the extract which occurs during elution. The first of these was not entirely overcome, and the sample used for infrared spectroscopy contained a large amount of inorganic perchlorate derived from buffer salt. Fortunately the perchlorate ion did not absorb in the critical portion of the spectrum and so did not interfere with the identification. In the purification procedure, high yields were regarded as secondary to the attainment of a homogeneous product (thus, for example, only the central portion of the propionylcholine peak in Fig. 2 was worked up); nevertheless, the amount of propionylcholine isolated (300 μg. from 10 kg. tissue corresponding to over 11% of the ethanol-extractable activity of the original spleen) does not seem unreasonable in view of the losses incurred in the reineckate precipitation and in the numerous assays, extractions and transfers. The yield of propionylcholine cannot be evaluated exactly owing to the difficulty of estimating the amount present in the original tissue, but Fig. 2 shows that about 60% of the total activity at this stage was due to propionylcholine and 30% to acetylcholine. Since propionylcholine is 1·5–2·0 times as active on the frog rectus as acetylcholine, this suggests that the extract contained at least as much propionylcholine as acetylcholine. If it is assumed that losses before chromatography affected both esters equally, the overall yield of propionylcholine might be as high as 20–25%.

Our estimate for the propionylcholine content of ox spleen is somewhat higher than that given by Banister *et al.* (1953). However, Banister (unpublished) has recently confirmed that part of the propionylcholine in spleen extracts may travel with acetylcholine on paper chromatograms, and the impression was also formed in the earlier work that propionylcholine (as the chloride) was less stable on paper chromatograms than was acetylcholine. Both these factors, which would lead to underestimation of the proportion of propionylcholine, are absent from the present work.

'F component' (Banister *et al.* 1953) was not detected in the effluents from the columns even after prolonged chromatography. However, some evidence was obtained that it was strongly adsorbed on the resin and could, like succinyldicholine and histamine, be eluted with acid. Work on the separation and identification of this substance is continuing.

The amount of 'acetylcholine' (i.e. total acetylcholine-like activity as assayed by the frog rectus) extracted from ox spleen by acid-ethanol in the experiments described in this paper was 1·6 units/g. tissue, equivalent to 0·3 μg. acetylcholine chloride/g. This is the same order of magnitude as has been observed before (0·3–0·9 μg./g.), but is much lower than that reported for horse and ox spleen by Chang & Gaddum (1933) (4–30 μg./g.), using the

trichloroacetic acid technique. Ambache (personal communication) has also obtained results lower than those reported by Chang & Gaddum. Differences in extraction procedure do not account for the discrepancy, as our values using trichloroacetic acid as the extractant were not more than 30 % higher than those obtained with acid-ethanol. Dale & Dudley (1929), using the acid-ethanol method, also reported high 'acetylcholine' levels in ox spleen, though their paper is somewhat vague as to the range of values actually found. Their range for horse spleen was 5–30 μg./g. (p. 101 of their paper); one batch of 32 kg. had 10·4 μg./g. (p. 111) and they state (p. 108), 'there is no reason for supposing that the yield...(of acetylcholine from ox spleen) would have been less under similar conditions'. However, it is possible to infer from an earlier passage (p. 99) that the acetylcholine content of a 50 kg. batch of ox spleen was only 2 μg./g. Their figures for horse spleen were confirmed by the isolation of acetylcholine from this tissue in relatively large quantities, but no such confirmation exists for ox spleen.

In order to exclude inadequacies in extraction technique as an explanation for the discrepancy between our own results and those of the Dale school, we have recently examined two horse spleens. Using the trichloroacetic acid method of extraction the 'acetylcholine' content was 6·8 and 7·6 μg./g. respectively; the acid-ethanol procedure gave results about 20 % lower (cf. Chang & Gaddum, 1933). These results are within the range given by Chang & Gaddum and are fairly close to the value reported by Dale & Dudley for their 32 kg. batch of spleen tissue. On submitting the active material to chromatography, almost 100 % of the activity behaved like acetylcholine on the column and in the subsequent differential assays. No other ester of choline was present in detectable amounts.

Aside from possible changes in diet, breeds and methods of slaughtering during the past 20 years, we believe that failure to allow for potentiating substances present in the crude tissue extracts may account, in part, for the high values of the earlier workers. We have found that, when no allowance is made for alkali-stable potentiating substances by the Feldberg technique, values nearly 3 times higher than the 'true' ones may be obtained. This would bring the upper part of our range near the lowest value given by Chang & Gaddum. By contrast, the amount of potentiation in the horse-spleen extracts was much less (16 and 0 % respectively). Now, neither Chang & Gaddum nor Dale & Dudley allowed for potentiating substances and in the case of the rabbit jejunum assays performed by the latter, one such substance, histamine, was admittedly present (they describe its isolation from ox spleen). We therefore believe our values represent the true ones obtainable under present-day conditions with currently acceptable assay techniques.

SUMMARY

1. The separation of propionylcholine from ox spleen by an improved method involving chromatography on ion-exchange resins is described.

2. The spleen propionylcholine was identified as such by its chromatographic and physiological properties and by its infrared absorption spectrum.

3. Reasons are advanced for believing that the values obtained for the total concentration of acetylcholine and acetylcholine-like substances in spleen tissue, though lower than those reported by earlier workers, represent the true values obtainable with modern assay techniques.

This investigation was supported by a research grant (H-1254) from the National Heart Institute of the National Institutes of Health, U.S. Public Health Service and by Hoffmann-La Roche Inc. We wish to express our gratitude to the following: the Rohm and Haas Company for gifts of resins, Herman Kemper & Sons for generous supplies of ox spleen, Mr D. Dalessandro for horse spleen, the May Institute of Medical Research for the use of their freeze-drying apparatus, the U.S. Public Health Service and Hoffman-La Roche, Inc. for financial support, Miss R. J. Banister and Mr I. A. Michaelson for help with some of the experiments.

REFERENCES

Balfour, W. E. & Hebb, C. (1952). *J. Physiol.* **118**, 94.
Banister, J., Whittaker, V. P. & Wijesundera, S. (1953). *J. Physiol.* **121**, 55.
Barer, R., Cole, A. R. H. & Thompson, H. W. (1949). *Nature, Lond.*, **163**, 198.
Berman, R., Wilson, I. B. & Nachmansohn, D. (1953). *Biochim. Biophys. Acta*, **12**, 315.
Chang, C. H. & Gaddum, H. J. (1933). *J. Physiol.* **79**, 255.
Dale, H. H. & Dudley, H. W. (1929). *J. Physiol.* **68**, 97.
Feldberg, W. & Hebb, C. (1947). *J. Physiol.* **106**, 8.
Gardiner, J. E. & Whittaker, V. P. (1953). *XIX Int. Physiol. Congr. Abstr.* p. 380.
Whittaker, V. P. (1953). *Biochem. J.* **54**, 660.
Whittaker, V. P. & Wijesundera, S. (1952). *Biochem. J.* **51**, 348.