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Isolation of Choline Esters from Aqueous Solutions by Extraction with Sodium Tetraphenylboron in Organic Solvents

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1. The method is based on the observation that choline esters and sodium tetraphenylboron (Kalignost) form complexes that are insoluble in water but soluble in organic solvents such as nitriles, higher ketones and benzyl alcohol. 2. The extraction procedure is an example of liquid cation exchange where tetraphenylboron is the cation-exchange group. 3. The proportion of choline esters extracted depends on the type and total amount of cation in the aqueous phase and the amount of sodium tetraphenylboron in the organic solvent. 4. The proportion of choline esters extracted is independent of the choline ester concentration, the pH (between 8 and 3) and the relative volumes of the two phases. 5. The affinity of sodium tetraphenylboron for choline esters increases with an increase in the size of the acyl group. 6. The choline ester extracted can be released into an aqueous solution by treatment with strong acids, silver salts and anion-exchange resins.

Owing to the small amounts of ACh* present in biological preparations and to its relative instability, the isolation of this compound is difficult. The methods generally employed for the isolation of ACh from dilute aqueous solutions include precipitation with ammonium reineckate (Kapfhammer & Bischoff, 1930), Na-TPB (Marquardt & Vogg, 1952) or aurichloride (Ewins, 1914). Column, paper and thin-layer chromatography or electrophoresis may be used to deal with more concentrated solutions (for literature review see Whittaker, 1963). Di-(2-ethylhexyl) hydrogen phosphate in chloroform has been used as an extractant, but few details are available (Johnston, Lloyd & Stone, 1968).

The problem of the isolation of ACh from dilute solutions has become increasingly important with the introduction of radioactively labelled ACh. This compound has become an important tool in studies on the uptake and release of ACh by tissues, slices (Schuberth & Sundwall, 1967) or synaptosome preparations (Marchbanks, 1968), and in radiochemical assays of choline acetyltransferase (Fonnum, 1966) or cholinesterases (Winteringham & Disney, 1964). Radioactively labelled compounds are measured with the highest efficiencies by liquid-scintillation counting. Many of the methods used today in the isolation of ACh are time-consuming and not very efficient for isolating it from dilute solutions. They are also not readily used in conjunction with liquid-scintillation counting since they often give rise to coloured precipitates and solutions or to dilute aqueous solutions of ACh.

The present method is based on the observation that the ACh–TPB complex is insoluble in aqueous solutions but soluble in some organic solvents such as nitriles and ketones. The effects of different ions, organic solvents, pH and variations in concentrations of Na-TPB and ACh on the extraction have been investigated. Very low concentrations of ACh could be isolated and any radioactivity was readily determined with high efficiency by liquid-scintillation counting. Procedures have been developed for further characterization of the extracted compounds by chromatography and electrophoresis. A preliminary account of this work has been published (Fonnum, 1968*a*).

* Abbreviations: ACh, acetylcholine; Na-TPB, sodium tetraphenylboron; TPB, tetraphenylboron.

MATERIALS AND METHODS

Materials. [1-^{14}C]ACh chloride, [*Me*-^{14}C]choline chloride and sodium [1-^{14}C]acetate were obtained from The Radiochemical Centre, Amersham, Bucks.; Na-TPB (Kalignost) was from E. Merck A.-G., Darmstadt, Germany; organic solvents were of the highest purity available and were from Koch–Light Laboratories Ltd., Colnbrook, Bucks. The Ringer–Locke solution contained (final concentrations): 150mM-NaCl, 5·5mM-KCl, 10mM-$NaHCO_3$, 2mM-$CaCl_2$ and 5mM-glucose.

Extraction procedures and assay of radioactivity. The extraction of ACh from an aqueous solution by organic

solvents was tested as follows (Tables 1, 2 and 3, and Figs. 1, 2 and 3). The organic solvent (1 or 2ml.) containing Na-TPB was added to an aqueous solution (usually 5ml.) that contained radioactive ACh and the ions under investigation. Twenty samples were shaken lightly by hand for a total time of 10min. and the phases separated by brief centrifugation (2000*g* for 2min.) in a swing-out head. Too vigorous shaking led to the formation of an emulsion of solvent in water, which collected as a milky layer at the interface between water and the organic solvent.

The distribution of ACh was determined from the distribution of radioactivity between the two phases. This was measured by scintillation counting in a Packard Tri-Carb model 3003 spectrometer. The scintillation mixture used for the non-aqueous phase consisted of 2ml. of acetonitrile and 10ml. of toluene containing 0·4% (w/v) 2,5-diphenyloxazole and 0·02% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. The radioactivity of the aqueous phase was determined by adding 0·5ml. of it to 5ml. of ethanol and 10ml. of the toluene scintillation mixture. The counting efficiencies for ^{14}C-labelled compounds in the presence of 1ml. of butyronitrile, benzyl alcohol and butyl ethyl ketone were 86, 80 and 72% respectively. The extraction procedure was highly reproducible, and results varied by less than 3%.

Other experiments were carried out to study the behaviour of the extracted ACh–TPB complex (Tables 4 and 5). In these experiments radioactively labelled ACh was dissolved in 40ml. of 10mM-sodium phosphate buffer, pH7·4, and shaken with 20ml. of organic solvent containing 100mg. of Na-TPB. The non-aqueous phase was isolated as before and divided into 20 samples. The samples were then treated with different aqueous solutions and assayed as before.

Assay for Na-TPB (*Kalignost*). The distribution of Na-TPB between aqueous and non-aqueous phases was determined from the absorption of Na-TPB in the far-u.v. region (195–210nm.) with a Perkin–Elmer 137 u.v. spectrophotometer. Reference samples were made up in phases treated similarly but without Na-TPB.

Electrophoresis and chromatography. Radioactive choline (5nmoles and 10^5c.p.m.) and ACh (5nmoles and 10^5c.p.m.) were extracted from 5ml. of Ringer–Locke solution into 1·0ml. of butyronitrile containing 20mg. of Na-TPB. Three samples were obtained and treated separately before they were subjected to electrophoresis or chromatography. (1) One sample was extracted in succession with 1ml. and 0·5ml. of 20% (w/v) trichloroacetic acid, which was then removed by extraction twice with 5ml. of ether. (2) One sample was treated with 0·5ml. of 2% (w/v) $AgNO_3$ solution and the excess of Ag^+ removed as chloride. (3) The third sample was treated with 2g. of drained Dowex 1 and 0·7ml. of 1mM-sodium phosphate buffer, pH5. The aqueous solutions from the treated samples contained more than 90% of the ACh and choline. Samples were subjected to paper electrophoresis, paper chromatography and column chromatography as described below. The paper chromatograms and electrophoretograms were scanned in a Packard model 701 radiochromatogram scanner. The samples from column chromatography were tested by liquid-scintillation counting with ethanol and toluene scintillation mixture.

Paper electrophoresis was carried out in 7·5% (v/v) acetic acid and 2·5% (v/v) formic acid in water as described by Potter & Murphy (1967). The paper chromatograms were developed with butan-1-ol–ethanol–acetic acid–water (8:2:1:3, by vol.) (Augustinsson & Grahn, 1953). Column chromatography was carried out with a 5ml. column of IRC-50 resin (Amberlite) equilibrated with 0·1M-NaH_2PO_4 at pH4·3 (Gardiner & Whittaker, 1954).

Ferric hydroxamate test. ACh extracted with 5mg. of Na-TPB in 1ml. of butyl ethyl ketone or butyronitrile was shaken with 0·5ml. of 2M-hydroxylamine hydrochloride in 3·5M-NaOH for 5min.; 0·25ml. of 4M-HCl and 0·25ml. of 0·2M-$FeCl_3$ were then added (Hestrin, 1949). The reaction took place in the aqueous phase only and the extinction was determined as usual. More than 90% of the ACh was recovered as acetohydroxamic acid by this procedure.

Stability of ACh. The radioactively labelled ACh was isolated and examined at different stages during the extraction procedures to ensure that it had not been broken down or hydrolysed. This was done by separating ACh by electrophoresis (for details see above) and comparing the radioactivity of the sample submitted for electrophoresis with that identified as ACh. In one experiment the ACh was extracted from 10mM-sodium phosphate buffer by Na-TPB in butyl ethyl ketone and in butyronitrile and left for 3 days at 5°. In all the cases examined, and also after storage for 3 days, more than 95% of the radioactive material was identified as ACh. Separate experiments showed that less than 0·1% of radioactive sodium acetate, the main radioactive hydrolysis product of ACh, was extracted by butyronitrile, butyl ethyl ketone or benzyl alcohol.

RESULTS

Extraction procedures. The preliminary experiments demonstrated that the proportion of ACh extracted from an aqueous solution was independent of the extraction method and of the proportion of ACh precipitated under similar conditions. More than 90% of ACh (2·5μmoles) dissolved in 1mM- or 100mM-sodium phosphate buffer (5ml.) could be precipitated by Na-TPB (14·6μmoles). If 1ml. of butyl ethyl ketone was added to the aqueous suspensions of ACh–TPB, the white precipitate disappeared and most of the ACh was extracted into the ketone phase. ACh was extracted to the same extent if Na-TPB was dissolved in the ketone first, to avoid any prior precipitation of ACh. In both extraction procedures 94% of the ACh was extracted from the weak phosphate buffer solution and 59% from the strong one. There was no increase in the proportion of ACh extracted after shaking for more than 3min. Similar proportions of ACh (92 and 54%) were left in the organic layer after ketonic phases containing ACh and Na-TPB had been washed with the two phosphate buffer solutions. ACh was not extracted by ketone in the absence of Na-TPB.

The distribution of Na-TPB between the two phases was examined spectrophotometrically. Only 1% of the Na-TPB was recovered in the strong phosphate buffer solution and 4% was left in the other buffer solution. With the strong phosphate buffer, less TPB (0·15μmole) than ACh (1·1μmoles)

Table 1. *Extraction of ACh from an aqueous solution by different solvents containing* Na-*TPB*

A portion (5ml.) of 50mM-NaCl solution that contained 2·5μmoles of ACh was extracted with 5mg. of Na-TPB dissolved in 1ml. (unless otherwise stated) of different organic solvents.

Solvent	Material extracted (%) ACh	Choline
Propionitrile	76	
Butyronitrile	79	
Vinylacetonitrile	92	63
Ethyl methyl ketone*	13	
Isopropyl methyl ketone*	18	6
Isobutyl methyl ketone	62	
Dipropyl ketone	80	
Butyl ethyl ketone	78	40
Di-isobutyl ketone	87†	
Butyl ethyl ketone–acetonitrile (5:1, v/v)	90	59
Benzyl alcohol	89	72
Butan-1-ol	26†	
Ethyl acetate	40	13

* 2ml.
† 0·25μmole of ACh

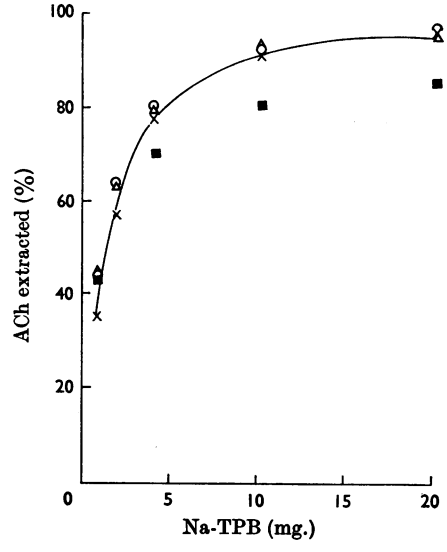

Fig. 1. Extraction of ACh by Na-TPB in 1ml. of butyl ethyl ketone at various concentrations of ACh. The symbols represent the percentage of ACh extracted from 5ml. of 40mM-NaCl solution containing: ○, 500nmoles; △, 5nmoles; ×, 0·05nmole; ■, 0·002nmole of ACh.

was therefore left in the aqueous phase. This ACh was not re-extracted by treatment with ketone alone. It was, however, precipitated by addition of Na-TPB or partially extracted by treatment once more with Na-TPB in ketone. Thus the ACh left in the aqueous phase after extraction by Na-TPB in ketone behaved as ACh ion and not as ACh–TPB.

Different extraction solvents. The extraction of ACh and choline by Na-TPB dissolved in different solvents is shown in Table 1. All the nitriles examined were excellent extraction solvents. They were readily separated from water, in which they were slightly soluble. The highest yield of extracted ACh was obtained with vinylacetonitrile; however, since this compound is slightly toxic it was considered unsuitable for routine use.

The ketones are another group of excellent solvents. The proportion of ACh extracted increased with increasing alkyl-chain length of the solvent, whereas the solubility of ACh–TPB decreased. Di-isobutyl ketone, which gave excellent results at a low ACh concentration (0·25μmole/5ml.), thus did not dissolve all the ACh–TPB complex at the high ACh concentration (2·5μmoles/5ml.). In the latter case the complex was left as a white precipitate at the water/ketone interface. It is noteworthy that a mixture of butyl ethyl ketone and acetonitrile gave better results than the ketone alone. This suggests a way of improvement of the available solvents.

Benzyl alcohol was an excellent extraction solvent; however, it is heavier than water, so the layer was sometimes difficult to separate from the bottom of a centrifuge tube. Butanol and ethyl acetate were not very efficient solvents for the extraction of ACh.

Several other solvents were examined and rejected. They included benzene, toluene, cyclohexane, *n*-hexane, cyclohexanone, acetophenone, diethyl ether, dichloroethane and carbon tetrachloride. Three solvents were selected for further investigations: butyl ethyl ketone, butyronitrile and benzyl alcohol.

Effect of pH. In this and subsequent experiments the conditions for the extraction of ACh were suboptimum for the Na-TPB concentration used, in order to detect any variation in proportion of ACh extracted. The extraction of ACh by Na-TPB (5mg.) in butyl ethyl ketone (1ml.) at different pH values in the range 3·0–8·0 was studied. The aqueous phase consisted of 100mM-sodium chloride in 10mM-sodium formate buffer (pH 4·5–3·0) or in 10mM-sodium phosphate buffer (pH 8·0–4·5). The proportion of ACh extracted (60–63%) was independent of pH within this range. The pK value of Na-TPB is below 1·5 (H. Saxholm, personal communication).

ACh concentration. The percentage of ACh extracted was independent of large variations in the ACh concentration in the aqueous phase (Fig. 1).

Table 2. *Effect of variations in volume of the aqueous phase on extraction of ACh*

ACh (10nmoles) was dissolved in different NaCl solutions and the solutions were each extracted with 5mg. of Na-TPB in 2ml. of butyl ethyl ketone.

Vol. of aqueous phase (ml.)	NaCl (M)	NaCl (μmoles)	Extracted ACh (%)
4·5	0·1500	675	60
15·0	0·0450	675	62
25·0	0·0270	675	62
50·0	0·0135	675	65
4·5	0·0450	203	82
4·5	0·0270	122	90
4·5	0·0135	61	96

The lowest concentration examined was 0·5pmole of ACh/ml., and this represents, not the limit of extraction, but the limit of detection of the radioactive ACh used (11mc/m-mole). The limit of extraction is probably far below this concentration.

Variations in the volume of the two phases. In the first experiments the volume of the ketone phase was varied from 0·3 to 3ml., but the amount of Na-TPB (5mg.) and the volume of the aqueous phase, 6ml. of 50mM-sodium phosphate buffer, were kept constant. The results showed that the same percentage of ACh (67–71%) was extracted in all cases and that the volume of the organic phase had no effect on the extraction.

In the second type of experiment a series of sodium chloride solutions containing ACh, of different volumes and concentrations, were treated with a constant volume of ketone containing Na-TPB (Table 2). The results showed that the proportion of ACh extracted depended on the total amount of sodium chloride present (column 3). No correlation was found between the percentage of ACh extracted and changes in volume alone (column 1) or molarities alone (column 2) of the sodium chloride solutions.

Effect of cations. The results in Table 2 demonstrated that the proportions of the ACh extracted depended on the total amounts of Na-TPB in the ketone phase and sodium chloride in the aqueous phase. It should therefore be possible to obtain a correlation between these three variables. The results showed that the proportion of ACh extracted decreased with an increase in the amount of sodium chloride in the aqueous phase, but increased with increasing amounts of Na-TPB in the organic phase (Fig. 2). It is therefore possible to extract nearly all the ACh from an aqueous phase by using a sufficiently large amount of Na-TPB. The experimental results gave a series of parallel straight lines when plotted as shown in Figs. 2(*a*) and 2(*b*). The results could therefore be expressed by the following equations:

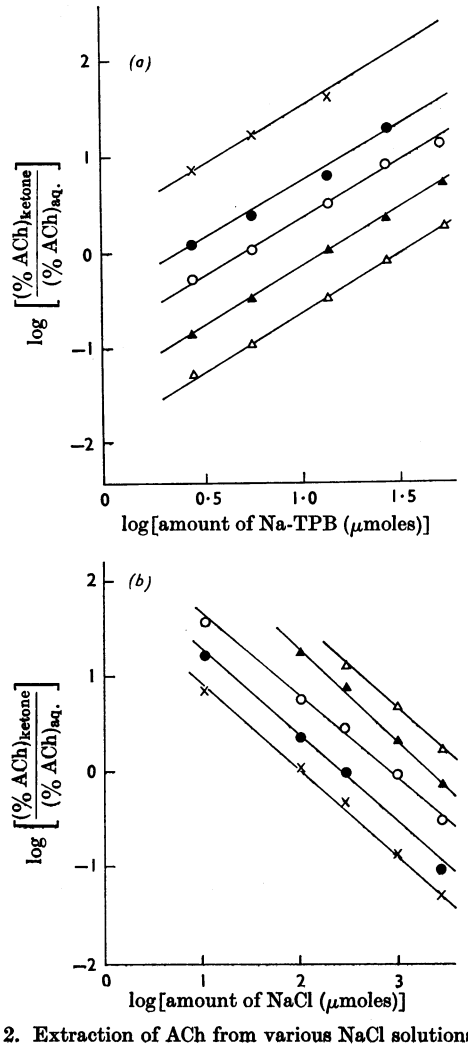

Fig. 2. Extraction of ACh from various NaCl solutions by various concentrations of Na-TPB in butyl ethyl ketone. The volume of the aqueous phase was 5ml. and that of the ketone phase 1ml. The initial concentration of ACh in the aqueous phase was 0·5nmole/ml. The logarithm of the ratio of ACh contents in the non-aqueous and aqueous phases is plotted as a function of the logarithm of (*a*) Na-TPB (μmoles/ml.) and (*b*) NaCl (μmoles/5ml.) concentrations. (*a*): ×, 10; ●, 100; ○, 300; ▲, 1000; △, 3000μmoles of NaCl/5ml. (*b*): ×, 2·9; ●, 5·8; ○, 14·5; ▲, 29·0; △, 58·0μmoles of Na-TPB/ml.

$$\log\left[\frac{(\%\ \mathrm{ACh})_{\mathrm{ketone}}}{(\%\ \mathrm{ACh})_{\mathrm{aq.}}}\right] = A_1 \log(\text{Na-TPB}) + C_1 = A_2 \log(\mathrm{NaCl}) + C_2 \qquad (1)$$

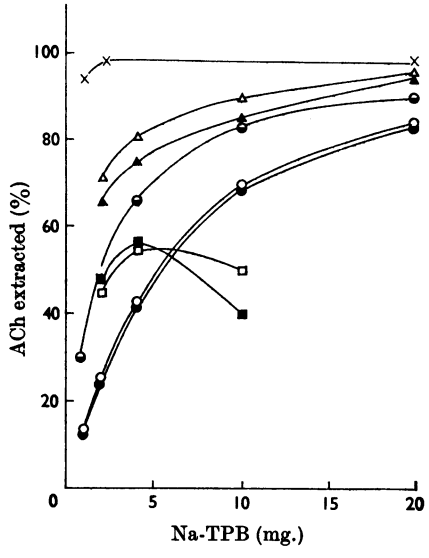

Fig. 3. Effect of sucrose and different salts on the extraction of ACh. The symbols represent the percentages of ACh extracted by Na-TPB in 1 ml. of butyl ethyl ketone from 5 ml. of an aqueous solution containing 2·5 nmoles of ACh and: ○, 1 m-mole of LiCl; ●, 1 m-mole of NaCl; ◒, 1 m-mole of tris–HCl buffer, pH 7·4; ▲, 1 m-mole of $CaCl_2$; △, 1 m-mole of $MgCl_2$; ×, 6 m-moles of sucrose; ■, 0·1 m-mole of KCl; □, 0·1 m-mole of NH_4Cl.

where the values in parentheses are the total amounts of Na-TPB and NaCl expressed in μmoles. From the slopes of the lines in Figs. 2(*a*) and 2(*b*), I found $A_1 = 1{\cdot}18 \pm 0{\cdot}04$ and $A_2 = -0{\cdot}94 \pm 0{\cdot}04$ respectively. The experimental results therefore satisfied the equation:

$$\frac{(\%\ \mathrm{ACh})_{\mathrm{ketone}}}{(\%\ \mathrm{ACh})_{\mathrm{aq.}}} = K \frac{[\mathrm{Na\text{-}TPB}]^{1{\cdot}18}}{[\mathrm{NaCl}]^{0{\cdot}94}} \qquad (2)$$

The constant K was found to be 71 ± 9. The theoretical basis for this relationship is dealt with in the Discussion section.

Alkaline-earth metal ions and tris–hydrochloric acid buffer interfered much less than Na^+ and Li^+ ions with the extraction of ACh (Fig. 3). These cations behaved as described for Na^+ ions and the results gave straight lines if plotted as in Fig. 2(*a*). K^+ and NH_4^+ presented a special problem since they had high affinity for TPB and their complexes with TPB were insoluble in water and only slightly soluble in the ketone. At low Na-TPB concentrations their complexes were still solubilized in the ketone and they did not interfere much with the extraction of ACh. At higher Na-TPB concentrations, however, their TPB complexes could no longer be completely dissolved in ketone and they were precipitated. This removed TPB ions as insoluble complexes with NH_4^+ and K^+ and therefore interfered strongly with the ACh extraction. The difficulties due to the presence of K^+ and NH_4^+ ions can be avoided by using other extraction solvents such as nitriles (Table 3) or mixtures of ketones and acetonitrile (Fonnum, 1968*a*). This is probably due to the higher solubilities of the TPB complexes in nitriles, since the results obtained at low Na-TPB concentrations were no better than those obtained with other solvents. Benzyl alcohol was unsuitable in the presence of these ions. There were some additional minor differences between the effects of the other cations, particularly tris–hydrochloric acid buffer, in the three standard solvents (Table 3).

Table 3. *Extraction of ACh from different salt solutions by Na-TPB in butyronitrile, benzyl alcohol and butyl ethyl ketone*

The aqueous phase (5 ml.) containing 2·5 nmoles of ACh and salts was extracted by Na-TPB in 1 ml. of organic solvent. The concentration of salts was 200 μmoles/ml. unless otherwise stated.

Na-TPB	ACh extracted (%)					
	Butyronitrile		Benzyl alcohol		Butyl ethyl ketone	
Salt (mg.) ...	2	10	2	10	2	10
LiCl	36	78	22	66	28	76
NaCl	33	71	39	80	27	75
Tris–HCl	39	80	22	65	50	84
$CaCl_2$	53	89	47	82	66	86
$MgCl_2$	60	96	49	78	76	90
KCl*	48	88	—	—	49	38
NH_4Cl*	44	97	—	—	45	50

* 20 μmoles/ml.

Effect of anions. In contrast with the effect of different cations, the proportion of ACh extracted was not markedly influenced by anions. The same percentage of ACh (74–77%) was extracted by 5 mg. of Na-TPB in 1 ml. of butyl ethyl ketone from solutions containing sodium fluoride, chloride, bromide, nitrate or phosphate when tested at the same Na^+ ion concentration (300 μmoles/5 ml.). A slightly higher extraction yield (80%) was obtained with sodium sulphate.

Extraction from sucrose and Ringer solutions. High sucrose concentrations, similar to those used in density gradients for the isolation of synaptosomes (Gray & Whittaker, 1962), had negligible effect on the proportion of ACh extracted (Fig. 3). The difficulties of isolation of small amounts of ACh from Tyrode and Ringer solutions could be overcome by selection of vinylacetonitrile as extraction solvent. Vinylacetonitrile containing 40 mg. of

Table 4. *Affinity of different compounds for Na-TPB in butyl ethyl ketone or butyronitrile*

Radioactively labelled ACh (50 μmoles) was extracted by 100 mg. (292 μmoles) of Na-TPB in 20 ml. of ketone or butyronitrile. The non-aqueous phase was separated into 20 samples and each was treated with 4 ml. of 10 mM-sodium phosphate buffer, pH 4·5, alone or together with 20 μmoles of one of the compounds listed below.

Compounds	Radioactivity recovered in non-aqueous phase (%)	
	Butyl ethyl ketone	Butyronitrile
Buffer alone	90	89
KCl	83	85
Carnitine chloride*	82	83
Phosphorylcholine chloride	84	85
Choline chloride	62	53
ACh chloride	36	32
Propionylcholine chloride	17	13
Butyrylcholine chloride	6	10
Benzoylcholine chloride	1	2
Acetylthiocholine chloride	—	11

* Neutralized carnitine hydrochloride.

Table 5. *Transfer of ACh from non-aqueous to aqueous phase*

ACh (20·0 nmoles) was extracted by 100 mg. of Na-TPB in 20 ml. of organic solvent. The non-aqueous phase was divided into 20 samples and tested with the aqueous solutions listed below.

Solution	Volume (ml.)	ACh recovered in aqueous phase (%)	
		From butyl ethyl ketone	From butyronitrile
Water	4·0	5	5
400 mM-HCl	1·0	95	75
100 mM-HCl	4·0	96	78
100 mM-HCl	1·0	79	67
400 mM-Formic acid	1·0	18	—
400 mM-Acetic acid	1·0	10	—
600 mM-Trichloroacetic acid	1·0	74	—
30 mM-$AgNO_3$	0·5	92	95
15 mM-$AgNO_3$	0·5	10	4
1 g. (dry wt.) of Dowex 1	1·0	92	90

Na-TPB extracted 88% of 1 nmole of ACh dissolved in 25 ml. of Ringer–Locke solution.

Affinities of carnitine and choline derivatives for TPB in an organic solvent. These experiments were based on the assumption that, if ACh–TPB in an organic solvent was treated with an aqueous solution containing an unlabelled compound capable of forming a complex with TPB, the proportion of radioactivity left in the non-aqueous phase would depend on the relative affinities of the two compounds for TPB. Carnitine, phosphorylcholine, potassium and choline had less affinity for TPB than had ACh (Table 4). Carnitine and phosphorylcholine do not form TPB complexes at this pH (4·0) owing to their negative charges. Separate experiments showed that radioactive acetylcarnitine may be isolated at about pH 2, when its carboxyl group is undissociated. Choline and potassium were also less readily extracted than ACh by Na-TPB in organic solvents (Tables 1 and 3).

The other choline esters investigated, including acetylthiocholine, all had higher affinities than ACh for Na-TPB, and the affinities of the esters correlated with their lipid solubilities. Higher choline esters, such as benzoylcholine, can therefore be used to displace ACh from a non-aqueous to an aqueous phase.

Isolation of ACh from the non-aqueous phase. Three methods were found suitable for removing ACh from the organic phase, namely treatment with strong acids, silver nitrate or anion-exchange resins (Table 5). The low pK value of Na-TPB makes it necessary to use strong acids to replace the choline esters by H^+ ions. ACh is reasonably stable in acid solution, the half-life in 0·4 M-hydrochloric acid being about 17 hr. (Bell & Robson, 1954). Acetic acid and formic acid were not suitable. ACh could also be removed from the organic phase by treatment with silver nitrate solution. Ag^+ ions formed with TPB a complex insoluble in aqueous, ketonic and nitrilic solutions. The precipitate was lighter than water, and after centrifugation the precipitate settled in the non-aqueous phase. Excess of Ag^+ ions in the aqueous solution were readily removed as silver chloride. The procedure was successfully carried out with a small volume of water.

Treatment of TPB with mercuric chloride gives phenylmercuric chloride (Wittig, Keicher, Rückert & Raff, 1949) and treatment of the organic phase by Hg^{2+} ions will therefore disrupt the complex and release ACh.

Anion-exchange resins have a high affinity for TPB. The organic phase was treated with a suspension of Dowex 1 in 1 mM-sodium phosphate buffer, pH 5. TPB was adsorbed by the ion-exchange resin, which was removed by filtration after 5 min. It was most important to drain the resin thoroughly to minimize the loss of water and thereby loss of ACh. Excellent recoveries were obtained by using glass filters and 1 ml. of aqueous solution.

Electrophoresis and chromatography. The procedures to release choline derivatives, previously extracted by a high concentration of Na-TPB in butyronitrile, were compared to find the best way of preparing an aqueous extract suitable for

electrophoresis or chromatography. Satisfactory separation of choline and ACh was achieved by electrophoresis, paper chromatography and column chromatography after treatment with silver nitrate solution or Dowex 1.

Separation by electrophoresis and by column chromatography after treatment with trichloroacetic acid was good but separation by paper chromatography was poor owing to the different choline salts giving double spots (Whittaker, 1963).

When a low TPB concentration (< 5mg.) was present in the ketone phase, this phase was treated with 1 drop of 6M-hydrochloric acid and separation of choline esters was achieved by electrophoresis by applying the mixture directly on to the paper.

DISCUSSION

The extraction procedure is an example of liquid cation exchange, TPB constituting the cation-exchanger. In this technique the ion-exchanger is dissolved in a water-immiscible phase and is then partitioned with an aqueous solution containing the ions to be separated. Liquid cation exchange was first applied in the isolation of anionic and cationic metal complexes (Coleman, Brown, Moore & Crouse, 1958; Blake, Baes & Brown, 1958), but has later been used in the isolation of nucleic acids and nucleotides (Khym, 1963), biogenic amines (Temple & Gillespie, 1966) and ACh (Johnston *et al.* 1968).

In several respects the extraction of choline esters by Na-TPB in organic solvents is analogous to their adsorption on cation-exchange resins. The proportion of choline esters extracted varies with the salt concentration (Fig. 2), the type of cation in the aqueous phase (Fig. 3), the hydrophobicities of the esters (Table 4) and the number of cation-exchange groups, in this case TPB molecules (Fig. 2).

Experiments showed that ACh left in the aqueous phase after extraction was present as ACh^+ ion and not as ACh–TPB. TPB, as a complex with Na^+ or ACh^+, was found to be present almost exclusively in the non-aqueous phase, whereas all the sodium chloride was assumed to be present in the aqueous phase. The exchange reaction taking place between ACh^+ ion and Na-TPB can be expressed as follows:

$$\text{Na-TPB} + \text{ACh}^+ \rightleftharpoons \text{ACh-TPB} + \text{Na}^+$$

By applying the law of mass action to the exchange reaction one gets:

$$K = \frac{\left(\frac{\text{Total amount of ACh–TPB}}{\text{Volume of organic phase}}\right)\left(\frac{\text{Total amount of Na}^+}{\text{Volume of aqueous phase}}\right)}{\left(\frac{\text{Total amount of ACh}^+}{\text{Volume of aqueous phase}}\right)\left(\frac{\text{Total amount of Na-TPB}}{\text{Volume of organic phase}}\right)} \quad (3)$$

Owing to the different solubilities of the organic solvents in water, the volume of the phases and consequently the molar concentrations of the substances could only be determined with difficulty. The volume terms cancel each other in eqn. (3) and consequently the total amounts of participants rather than molar concentrations were used to calculate K. The equation is similar to that obtained for ion-exchange resins (Kitchener, 1957).

The amounts of ACh used in the experiments were small compared with those of the sodium salts. The amount of Na-TPB was therefore assumed to be equal to the total amount of TPB present. Similarly, the amount of Na^+ was equal to that derived from the salts present in the aqueous phase. The amounts of ACh–TPB and ACh^+ might be expressed as the percentages of ACh recovered in the non-aqueous and aqueous phases respectively. Eqn. (3) could therefore be modified to give:

$$\frac{(\%\ \text{ACh})_{\text{non-aq.}}}{(\%\ \text{ACh})_{\text{aq.}}} = K\frac{(\text{Total amount of Na-TPB})^{a_1}}{(\text{Total amount of NaCl})^{a_2}} \quad (4)$$

Since the values for Na-TPB and sodium chloride are approximations, two correction exponents, a_1 and a_2, which must be near unity, were introduced. The equation now agrees with the experimental results (Fig. 2). From the data derived from Fig. 2, $K = 71$, $a_1 = A_1 = 1{\cdot}18$ and $a_2 = -A_2 = 0{\cdot}94$.

The affinities of different compounds for Na-TPB as a cation-exchange group were extrapolated from the results in Fig. 3 and Table 4. The order of affinities was sucrose $< Mg^{2+} < Ca^{2+} <$ tris–hydrochloric acid buffer $< Li^+ = Na^+ < K^+ = NH_4^+ \ll$ choline < ACh < propionylcholine < acetylthiocholine < butyrylcholine < benzoylcholine. This order of affinities is valid for TPB in butyronitrile and butyl ethyl ketone, but benzyl alcohol gives slightly different results for the Na^+ ion (Table 3). The series of affinities is valid only when the solubility of the TPB complexes in the non-aqueous phase is not exceeded.

The choline esters show a similar increase in order of affinity for a cation-exchange resin (Whittaker, 1963), probably due to an increase in van der Waals attraction forces.

In the series of solvents examined, e.g. ketones, the most efficient were those least soluble in water (Table 1). The less water-soluble ketones probably maintain the highest proportion of TPB complex in

the non-aqueous phase. The most efficient extraction solvent found was vinylacetonitrile; however, butyronitrile and butyl ethyl ketone sufficed for most purposes.

The extraction method compares favourably with the precipitation methods available for the isolation of ACh. Considerably lower concentrations of ACh could be isolated by extraction with Na-TPB in an organic solvent than by precipitation with Na-TPB, where the concentration limit of ACh is 50 μg./ml. (Marquardt & Vogg, 1952). Choline esters may, in part, be selectively extracted by controlling the variable extraction conditions, such as concentrations of TPB and salts and pH, whereas precipitation gives mixed precipitates of the esters (Marquardt & Vogg, 1952).

Other compounds such as ammonium reineckate, dipicrylamine and di-(2-ethylhexyl) hydrogen phosphate may replace TPB as the cation-exchange group. The extraction of ACh from sodium chloride and potassium chloride solutions by these three compounds was examined, but all three were inferior to Na-TPB. At conditions where di-(2-ethylhexyl) hydrogen phosphate recovered 80% of the ACh (Johnston *et al.* 1968), Na-TPB extracted 99%. The present report seems to be the first application of Na-TPB in liquid cation exchange, and this compound may be of general use in the isolation of other basic compounds under suitable conditions.

The choline esters extracted could also be determined directly by the ferric hydroxamate test (Hestrin, 1949). After release of the extracted compounds into aqueous solution, their concentration could be determined by biological assay with guinea-pig ileum. Traces of organic solvent, particularly nitrile, interfered with the assay, but could be removed by extraction with ether.

The compounds released into aqueous solution could be characterized further by electrophoresis or chromatography. The chromatographic separation of TPB complexes (Augustinsson & Grahn, 1954) was unsuccessful under my conditions owing to the large excess of Na-TPB in the non-aqueous phase. The extraction procedure has been used in the assay of soluble and membrane-bound choline acetyltransferase (Fonnum, 1968*b*), and has also been successfully applied in sensitive assay methods for choline acetyltransferase (sensitivity: 0·1 μg. wet wt. of brain) and acetylcholine esterase (sensitivity: 5ng. wet wt. of brain) (F. Fonnum, unpublished work).

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