Physical Studies of the Interactions of Acetylcholine Chloride with Membrane Constituents

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The binding of acetylcholine to pure lipids, and lipids, proteins and lipoproteins extracted from synaptic membranes, was investigated by monolayer and n.m.r. techniques. No specific binding of acetylcholine could be detected at the concentration used, although its muscarinic and nicotinic antagonists [atropine and (+)-tubocurarine respectively] could be shown to interact with the membrane components. It is concluded that the binding of the nicotinic and muscarinic antagonists of acetylcholine is not necessarily indicative of the existence of a specific acetylcholine receptor. Measurements of the displacement of $^{45}\text{Ca}^{2+}$ from monolayers of phosphatidylserine by acetylcholine and the variation of electrophoretic mobility of phosphatidylserine particles with concentration of acetylcholine indicated that in these systems acetylcholine was acting as a counterion at the negatively charged lipid interface. But studies of the salting-in and salting-out of negatively charged lipid aggregates showed that acetylcholine and other quaternary ammonium compounds did not here behave simply as counterions. Electrostrictively hydrated cations such as Na^+ and K^+ were found to salt out, whereas hydrophobically hydrated cations such as acetylcholine salted in such aggregates. The possible role of the hydration of acetylcholine in synaptic transmission is discussed.

It is now well established that the acetylcholine cation acts as a neurotransmitter at certain synapses in the central and peripheral nervous system (Hebb & Krnjević, 1962). It has been suggested that acetylcholine acts to induce a conformational change in the membrane that leads to depolarization by causing a change in the ionic permeability of the postsynaptic membrane. To explain the observed structural specificity of many agonists and antagonists at the cholinergic synapse (Barlow, 1955) it has been postulated that a chemical receptor with a well-defined configuration exists in the postsynaptic membrane. However, the unambiguous isolation in a pure state of the acetylcholine receptor in such a way as to preserve the stereochemical specificity observed *in vivo* has not so far been achieved.

Although the putative acetylcholine receptor has been investigated there have been up to now only limited attempts to explore the physical chemistry of the acetylcholine cation itself. This is a major lacuna in the field, since many of the above conclusions about the receptor rest on the results of experiments with agonist and antagonist substances. These compounds often have grossly

dissimilar structures, so that their various effects may depend not only on the specific requirements of the receptor but also on the different physical properties of the agonist and antagonist. This paper therefore presents results for the binding of acetylcholine to various membrane constituents in some model systems.

EXPERIMENTAL

Materials

Synaptic vesicles and the external membrane of the nerve-ending terminal (synaptosome membranes) were isolated from scraped guinea-pig cerebral cortex as described by Whittaker, Michaelson & Kirkland (1964). Postsynaptic membranes were isolated by Triton X-100 treatment of nerve-terminal membranes as described by Lapetina & De Robertis (1968). Approximate values for the lipid content of fractions isolated from 1 g wet wt. of cortex are 0.089, 0.9 and 0.53 mg of lipid in synaptic vesicles, synaptosome membranes and postsynaptic membranes respectively, and for protein 0.81, 0.80 and 2.76 mg respectively.

The lipids of synaptic vesicles, synaptosomes and postsynaptic membranes were extracted with chloroform-methanol (2:1, v/v) as described by Eichberg, Whittaker

& Dawson (1964). The residual membranes after the extraction of lipids were treated with water to extract the membrane protein. The aqueous solution of the proteins was separated from insoluble material by centrifugation.

Egg-yolk lecithin was prepared from fresh egg yolks by the method of Singleton, Gray, Brown & White (1965). (Dimyristoyl), (dipalmitoyl) and (distearoyl) phosphatidylcholine and (dimyristoyl) phosphatidylethanolamine were synthesized and characterized as described by Phillips & Chapman (1968). (Distearoyl) phosphatidic acid was synthesized in this laboratory by Dr A. Davies. It was chromatographically pure with m.p. 70–72°C and $[\alpha]_D^{25} +3.5^\circ$ (in chloroform). Ox brain phosphatidylserine was obtained from Lipid Products Ltd., Epsom, Surrey, U.K. It was purified on silicic acid (Long, Shapiro & Staples, 1962) and used as the monosodium salt. Phosphatidylinositol was extracted from frozen peas and purified on DEAE-cellulose by following methods described by Rouser, Kritchevsky, Heller & Lieber (1963) and Ansell & Hawthorne (1964). Sphingomyelin, cerebroside, cardiolipin and stearic acid were obtained from Applied Science Laboratories Inc., State College, Pa., U.S.A., and used without further purification. Monocetyl dihydrogen phosphate was obtained from Albright and Wilson Ltd., Birmingham, U.K. Cholesterol was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and purified by recrystallization. Ox brain phosphatidic acid was a gift from Mr N. Miller (Agricultural Research Council, Babraham, Cambridge, U.K.) and triphosphoinositide and ox brain ganglioside (28% *N*-acetylneuraminic acid) were generously provided by Dr R. M. C. Dawson (Agricultural Research Council, Babraham, Cambridge, U.K.). T.l.c. on silica gel H (E. Merck A.-G., Darmstadt, Germany) with chloroform-methanol-aq. 7*M*-ammonia (46:18:3, by vol.) as the solvent gave single spots with all the lipids.

The remaining chemicals were all obtained from commercial sources. Acetylcholine chloride was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. The purities of this and the labelled compound described below were checked by t.l.c. on MN cellulose 300H (Macherey, Nagel und Co., 516 Düren, Germany) (Marchbanks, 1968) and by n.m.r. spectroscopy. Both methods showed that the samples were free of choline chloride. [*acetyl*-1-¹⁴C]choline chloride (specific radioactivity 60 μ Ci/mg) and ⁴⁵CaCl₂ (specific radioactivity 2–10 mCi/mg of Ca) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [*carboxy*-¹⁴C]-Procaine hydrochloride (specific radioactivity 10.4 μ Ci/mg) was obtained from NEN Chemicals G.m.b.H., Dreieichenhain, Germany. The water used was laboratory-distilled water, deionized and redistilled from alkaline permanganate under N₂ in an all-glass apparatus (Phillips & Chapman, 1968).

Methods

Monolayers. A Teflon trough (capacity 85 ml, plus approx. 15 ml for meniscus) similar to that described by Hauser & Dawson (1967) was used. The surface pressure was measured with a Wilhelmy plate suspended from the arm of a recording Cahn micro-balance (Cadenhead, Demchak & Phillips, 1967). The radioactivity technique used was as described by Hauser & Dawson (1967, 1968). A gas-flow counter (Actigraph 3 detector chamber from

Nuclear-Chicago) covered with a 4.3 cm × 1.4 cm window (Micromil window 150 μ g/cm², from Nuclear-Chicago) was used.

The lipid mixtures extracted from natural membranes and the pure lipids were evaporated to dryness and dissolved in hexane-ethanol from which they were spread at the air/water interface (Phillips & Chapman, 1968). The membrane proteins were spread from aqueous solution by carefully running the solution down a glass rod on to the water surface (Gaines, 1966). The total lipoproteins of the natural membranes were spread at the air/water interface from 2-chloroethanol, dimethylformamide or a mixture of both. The washed pellet from each membrane dispersion was dispersed in 0.2 ml of water. Addition of 0.8 ml of one of the above organic solvents dissolved the membrane fraction completely and the membrane lipoproteins were spread from this solution.

Electrophoresis. The electrophoretic mobilities of phosphatidylserine particles dispersed in an aqueous medium were measured in a cylindrical horizontal electrophoresis cell as described by Bangham, Flemans, Heard & Seaman (1958). The electrophoretic mobilities measured at 25 ± 0.05°C were reproducible to within ±3%. The monosodium salt of phosphatidylserine (approx. 3 mg) dissolved in chloroform-methanol (8:1, v/v) was evaporated to dryness *in vacuo* and 5 ml of a solution containing the compounds listed in Fig. 1 and Table 2 in the appropriate concentration was added. The phosphatidylserine was dispersed by shaking for 15 min, until no phospholipid could be seen adhering to the glass wall of the test tube. The pH values of the solutions varied in the range 5–6.

Qualitative precipitation experiments. Egg-yolk lecithin or the monosodium salt of phosphatidylserine dissolved in chloroform-methanol (8:1, v/v) was evaporated to dryness *in vacuo*. Water (1 ml/20 mg of phospholipids) at pH 5.5 was added and the phospholipid dispersed as described above. The aqueous dispersions of lecithin and phosphatidylserine were ultrasonically treated at 20 kHz (low-power level) with a Kerry Vibrasom System 150 (Kerry Ultrasonics Ltd., Hitchin, Herts., U.K.) for 2–5 min. Sodium dodecyl sulphate was dissolved in water to give solutions containing 20 mg/ml. The compounds listed in Table 3 were either added as solids or as concentrated solutions to the clear aqueous phospholipid dispersions or the micellar solutions of sodium dodecyl sulphate.

Quantitative precipitation experiments. Known amounts of the compounds listed in Table 2 were added to 2% (w/v) phosphatidylserine dispersions either as solids or as concentrated aqueous solutions. The extinctions of the phosphatidylserine dispersions were determined as a function of the concentration of additive at 520 nm in a Unicam SP.500 spectrophotometer.

N.m.r. spectroscopy. N.m.r. spectra were obtained at 33°C on a Perkin-Elmer model R10 spectrometer operating at 60 MHz. With low concentrations of solutes a signal averager (Northern Scientific N.S. 544, digital memory oscilloscope) was used to increase the signal/noise ratio. The phosphatidylserine dispersions containing 10 mg/ml were prepared as described above, except that D₂O was used instead of water. The membrane fractions were dispersed in sodium-potassium phosphate buffer, pH 7.2 and 10.2, at concentrations of 0.4–2 mg of protein/ml. They were mixed with acetylcholine chloride

so that the final concentration was 0.1M with synaptic vesicles and synaptosome external membranes, and 0.05M with postsynaptic membranes.

RESULTS

Monolayers. To obtain qualitative information on the binding of acetylcholine to ordered arrays of lipid polar groups the increase in surface radioactivity was measured when insoluble monolayers were spread to film pressures (π) of 5–20 dyn/cm on water at pH 5.5 containing ^{14}C -labelled acetylcholine chloride at a concentration of approx. $1\text{ }\mu\text{M}$. No significant increase in surface radioactivity was detected when the monolayers were formed from (dimyristoyl), (dipalmitoyl) or (distearoyl) phosphatidylcholines, (dimyristoyl) phosphatidylethanolamine, phosphatidylserine, sphingomyelin, cardiolipin, ganglioside, cerebroside, stearic acid or cholesterol. The only phosphate diester that gave a slight increase in surface radioactivity was phosphatidylinositol at 30 dyn/cm. However, a significant increase in surface radioactivity was obtained when phosphatidylserine was spread (π approx. 20 dyn/cm) on water containing $4.4\text{ }\mu\text{M}$ -[carboxy- ^{14}C]procaine hydrochloride.

In contrast, lipid molecules containing a monoester of phosphoric acid under the experimental conditions did bind acetylcholine. Increases in surface radioactivity of 5–15 counts/s were observed with monolayers of ox brain and (distearoyl) phosphatidic acids, triphosphoinositides and monocetyl phosphate (although the last-named desorbed from the interface quite rapidly and a pressure greater than approx. 1 dyn/cm was not attained). This

increase of 5–15 counts/s arises when one acetylcholine cation is distributed with about ten lipid molecules. No significant changes in π due to the presence of acetylcholine were detected in any of the above experiments. When the ionic strength of the substrate was increased (e.g. 0.1M-sodium chloride) no significant adsorption of acetylcholine occurred with any of the above lipids.

Exactly analogous experiments were performed with monolayers of the total lipids, the proteins and total lipoprotein of synaptic vesicles, synaptosome external membranes and postsynaptic membranes. None of these bound labelled acetylcholine. The binding of acetylcholine to the natural membranes was also studied by n.m.r. spectroscopy. No significant change in the acetylcholine spectrum could be detected in the presence of the natural membranes except that some of the acetylcholine was hydrolysed in the presence of postsynaptic membranes, giving rise to an acetate signal. In contrast with acetylcholine, the line widths in the spectra of local anaesthetics (procaine hydrochloride, tetracaine hydrochloride), atropine sulphate and (+)-tubocurarine were significantly increased in the presence of postsynaptic membranes.

To obtain quantitative information on the binding of acetylcholine the concentrations of various additives required to displace $^{45}\text{Ca}^{2+}$ from phosphatidylserine monolayers were examined (Table 1). Acetylcholine appears to behave simply as a small univalent cation. The requisite concentrations of the pharmacologically active bases were one, two or three orders of magnitude less, depending on the intrinsic surface-activity of the added compound. When $^{45}\text{Ca}^{2+}$ was displaced with non-surface-active

Table 1. Concentrations of additive in the Langmuir trough required to decrease the surface radioactivity of bound $^{45}\text{Ca}^{2+}$ twofold

The monomolecular film was ox brain phosphatidylserine maintained at 15–20 dyn/cm ($65\text{--}75\text{ }\text{\AA}^2/\text{molecule}$). The concentration of $^{45}\text{CaCl}_2$ in the bulk phase was approx. $0.1\text{ }\mu\text{M}$. The first seven compounds listed are not surface-active. The surface tensions of $100\text{ }\mu\text{M}$ solutions of procaine hydrochloride and (+)-tubocurarine hydrochloride are about 1 dyn/cm less and the surface tension of a $10\text{ }\mu\text{M}$ solution of cetyltrimethylammonium bromide is about 10 dyn/cm less than that of water.

Compound (anion Cl^- unless stated otherwise)	Approx. concn. of substrate required to displace 50% of $^{45}\text{Ca}^{2+}$ (μM)	Approx. increase in monolayer pressure ($\Delta\pi$) (dyn/cm)
Na^+	1500	
K^+	1500	
NH_4^+	2000	
Tetramethylammonium	3500	
Tetraethylammonium iodide	500	
Acetylcholine	1500	
Noradrenaline bitartrate	100	<1
Procaine	35	5
Atropine sulphate	10	5
(+)-Tubocurarine	3	10
Cetyltrimethylammonium bromide	0.5	20

compounds no significant changes in the monolayer pressure were observed. With the surface-active bases the increase in π was in the range 5–20 dyn/cm (Table 1).

Dispersed systems. Phospholipids can be dispersed in water to give ordered structures (Luzzati, 1968; Chapman, Williams & Ladbroke, 1967) of which, in particular, the liquid crystalline phase consisting of particles or liposomes comprised of bimolecular lamellae (Bangham, 1968) is a convenient model for studying ion-binding to lipid polar groups.

Electrophoresis. Fig. 1 shows the variation in

Fig. 1. Variation of electrophoretic mobility of phosphatidylserine particles dispersed in water as a function of solute concentration. ▲, Tetramethylammonium chloride; □, acetylcholine chloride; ○ and ●, NaCl and KCl; Δ, procaine hydrochloride; ■, atropine sulphate; ▽, CaCl_2 .

electrophoretic mobility of phosphatidylserine particles as a function of additive concentration. The acetylcholine and tetramethylammonium cations were only slightly less effective in decreasing the electrophoretic mobility than the univalent inorganic cations, the effect of which was in the order $\text{Li}^+ > \text{Na}^+ > \text{NH}_4^+ > \text{K}^+$. The bivalent cations Ca^{2+} and Mg^{2+} were much more effective than the univalent ions, Ca^{2+} being somewhat more effective than Mg^{2+} . The pharmacologically active bases procaine and atropine both decreased the electrophoretic mobility much more effectively than acetylcholine. Cetyltrimethylammonium bromide and (+)-tubocurarine at concentrations of about 1 mM decreased the electrophoretic mobility of phosphatidylserine particles in 0.02 M potassium chloride virtually to zero. Plotting the electrophoretic mobilities against the logarithm of additive concentration gave straight lines that could be extrapolated to give the concentrations required to decrease the electrophoretic mobility to $(-)$ $1 \mu\text{s}^{-1} \text{V}^{-1} \text{cm}^{-1}$ (Table 2).

Precipitation. The qualitative effects of adding various cations on the precipitation of aqueous 2% dispersions of phosphatidylserine are summarized in Table 3. Inorganic cations caused precipitation, whereas, in contrast, the solution remained clear when high concentrations (several molar) of the alkylated ions were added (Table 3, part 3). The biologically active compounds also exhibited this exceptional behaviour (Table 3, part 1). The alkylammonium ions with more than seven carbon atoms form micelles and were able to redissolve the precipitate when added in excess. Analogous results to those presented in Table 3 were obtained with 2% micellar solutions of sodium dodecyl sulphate in water. In contrast, aqueous 2% dis-

Table 2. Correlation between electrophoresis and turbidity results for phosphatidylserine dispersions

Compound (anion Cl^- unless stated otherwise)	Concn. required to decrease electrophoretic mobility to $(-)$ $1 \mu\text{s}^{-1} \text{V}^{-1} \text{cm}^{-1}$ (M)	Concn. required to bring E_{520} to 2 (M)
K^+	0.7	1.4
Na^+	0.7	1.4
Acetylcholine	0.85	No precipitation
Tetramethylammonium	1.2	No precipitation
Ca^{2+}	0.006	0.009
Procaine	0.2	0.25
Noradrenaline bitartrate	0.2	0.25
Atropine sulphate	0.1	No precipitation
Cetyltrimethylammonium bromide*	0.0012	0.006
(+)-Tubocurarine*	0.0009	0.009

* The discrepancy observed with cetyltrimethylammonium bromide and (+)-tubocurarine arises because the electrophoresis experiments were carried out in the presence of 0.02 M-KCl whereas the precipitation experiments were done in the absence of salt. For the electrophoresis experiments with cetyltrimethylammonium bromide and (+)-tubocurarine it was necessary to add KCl to make the phosphatidylserine particles visible.

Table 3. *Effects of additives on the solubility of 2% dispersions of the monosodium salt of phosphatidylserine in deionized water*

	Compound (anion Cl ⁻ unless stated otherwise)	Precipitation
1	Acetylcholine	—
	Choline bromide	—
	Atropine sulphate	—
2	Li ⁺	+
	Na ⁺	+
	K ⁺	+
	Mg ²⁺	+
	Ca ²⁺	+
	NH ₄ ⁺	+
3	Methylammonium	—
	Dimethylammonium	—
	Trimethylammonium	—
	Tetramethylammonium	—
	Ethylammonium	—
	Diethylammonium	—
	Triethylammonium	—
	Tetraethylammonium	—
	Propylammonium	—
	Tetrapropylammonium	—
	Butylammonium	—
	Tetrabutylammonium	—
4	Pentylammonium	+
	Hexylammonium	+
	Heptylammonium	+
	Octylammonium	+
	Nonylammonium	+
	Decylammonium	+
	Dodecylammonium	+
	Hexadecylammonium	+
	Cetyltrimethylammonium bromide	+
	Procaine	+
	Noradrenaline bitartrate	+
	(+)-Tubocurarine	+

pensions of egg-yolk lecithin were not precipitated by compounds listed in Table 3.

Consistent with the precipitation experiments the line widths of the acetylcholine and tetramethylammonium n.m.r. signals were not significantly changed in the presence of phosphatidylserine particles, whereas the line widths of procaine hydrochloride (Hauser, Penkett & Chapman, 1969b) and atropine sulphate were broadened, indicating electrostatic interactions with the head-group region of phosphatidylserine. Also, cetyltrimethylammonium bromide, which was completely incorporated into phospholipid bilayers (E. G. Finer & H. Hauser, unpublished work), showed strong electrostatic interaction in the head-group region with little effect on the molecular motion of the fatty acid-chain region (Phillips, Williams & Chapman, 1969).

Quantitative information on the relative effectiveness of the various cations in precipitating aqueous

2% dispersions of phosphatidylserine is summarized in Table 2. The concentrations required to bring the extinction at 520nm to a fixed value of 2 parallel the concentrations required to decrease the electrophoretic mobility of phosphatidylserine particles to (—) 1 $\mu\text{ms}^{-1}\text{V}^{-1}\text{cm}^{-1}$ (Table 2) for all compounds except acetylcholine, tetramethylammonium and atropine.

It is clear from these experiments that acetylcholine and the short-chain quaternary ammonium ions behave purely as counterions in the electrical double layer. However, although their electrical properties are almost the same as the simple inorganic univalent cations, they do not precipitate negatively charged lipid particles, and therefore additional reasons must be sought for this anomalous behaviour.

DISCUSSION

Monolayers. With most of the lipid monolayers tested no significant binding of ¹⁴C-labelled acetylcholine was observed at acetylcholine concentrations of 1 μM . The binding of ⁴⁵Ca²⁺ to acidic phospholipids (Hauser, Chapman & Dawson, 1969a) and of ¹⁴C-labelled cetyltrimethylammonium bromide to phosphatidylinositol (Hauser & Dawson, 1968) clearly shows that measurement of the surface radioactivity is sensitive enough to detect any strong binding. Thus, in contrast with ¹⁴C-labelled acetylcholine chloride, a significant increase in surface radioactivity was observed with [*carboxy*-¹⁴C]procaine hydrochloride, which has been shown to bind to negatively charged phospholipids by Hauser & Dawson (1968) and Hauser *et al.* (1969b). Monolayers of phospholipids containing monoesterified phosphate groups gave rise to measurable increases of the surface radioactivity above the background. This probably arises because the number of negative charges per phospholipid molecule is increased, although with phosphatidic acid and monocetyl phosphate this would require the phosphate group to be fully ionized at pH 6 (cf. Hauser & Dawson, 1967). Electrostatic forces appear to control the interaction and such non-specific effects would be observed with any positively charged ion. Acetylcholine seems to act merely as a counterion and remains exclusively in the double-layer region since only small changes in π were observed and no adsorption of acetylcholine occurred in the presence of 0.1M-sodium chloride. In this case the Na⁺ concentration exceeds that of acetylcholine 10⁵-fold and, in the absence of any specific binding, the acetylcholine in the double layer is displaced by Na⁺. Our results are incompatible with the suggestion that the electrically neutral phosphatidylcholine can bind acetylcholine (Burton & Howard, 1967).

From our results with pure lipid monolayers it is not surprising that monolayers of mixed lipids extracted from natural membranes containing a relatively small percentage of negatively charged phospholipids showed no rise in surface radioactivity when ^{14}C -labelled acetylcholine was added to the bulk phase. Similarly, monolayers composed of proteins and lipoproteins derived from biological membranes that are likely to contain cholinergic receptors failed to disclose any specific binding of acetylcholine. The monolayer model might be unlikely to demonstrate a specific drug-receptor interaction because the requisite natural conformations might have been destroyed during the various experimental manipulations. However, the model does demonstrate the difference in behaviour of acetylcholine and its nicotinic and muscarinic antagonists [(+)-tubocurarine and atropine]. The fact that in both the monolayer and n.m.r. experiments acetylcholine could not be shown to interact with membrane components whereas atropine and (+)-tubocurarine did suggests that these substances display interactions that are not of themselves indicative of the existence of the acetylcholine receptor. The extent to which this interaction of atropine and (+)-tubocurarine with membrane components plays a role in their pharmacological activity is uncertain, but it is clear that this phenomenon must be borne in mind in interpreting their cholinergic antagonist effects and in using the binding of these substances as a model of the interaction of acetylcholine with its receptor.

Table 1 shows that small ions generally require a substrate concentration of the order of millimolar to displace half of the $^{45}\text{Ca}^{2+}$ bound to a phosphatidylserine monolayer under the experimental conditions used. Lower concentrations of compounds like procaine hydrochloride, noradrenaline bitartrate or atropine sulphate suffice because they contain, in addition to positively charged alkylammonium groups, other groups such as benzene rings that contribute to the adsorption free energy. The attraction of these molecules is probably initiated by electrostatic forces, which bring the oppositely charged molecules in close contact with each other enabling shorter-range forces (dipole forces and Van der Waals forces) to become effective (Hauser *et al.* 1969b). As a result, these molecules are oriented at the lipid/water interface of phosphatidylserine monolayers with the more hydrophobic parts of the molecule penetrating to various degrees into the region of low dielectric constant. This causes pressure increases (Table 1) that depend on the size, shape and orientation of the penetrated molecule and initial monolayer pressure and phase (Hauser & Dawson, 1968). With the highly surface-active long-chain quaternary ammonium compound cetyltrimethylammonium bromide the quaternary

ammonium group is presumably oriented close to the negative charge of the phosphatidylserine molecule with the alkyl chain interacting with the alkyl chains of the phosphatidylserine molecules. In addition to electrostatic and Van der Waals forces a hydrophobic interaction term contributes to the adsorption free energy. This additional interaction energy is reflected in the highest $\Delta\pi$ obtained with cetyltrimethylammonium bromide. (+)-Tubocurarine is particularly effective because it contains two positively charged alkylammonium groups.

Dispersed systems. Since the electrophoretic mobility is proportional to the ζ -potential, the results in Fig. 1 give quantitative information on how the ζ -potential, and thus the double-layer potential (Davies & Rideal, 1963), changes with concentration of added ions. The electrostatic repulsive energy between the phosphatidylserine colloidal particles is proportional to the ζ -potential, and, when the value of the latter is such that the electrophoretic mobility is $\lesssim (-) 1 \mu\text{ms}^{-1} \text{V}^{-1} \text{cm}^{-1}$, the adhesion between the particles becomes great enough for flocculation to occur. Thus a correlation between the electrophoresis and turbidity results of Table 2 is to be expected if electrostatic effects control the flocculation. All the compounds of Table 2 except tetramethylammonium, acetylcholine and atropine showed parallel behaviour in the electrophoresis and precipitation experiments. If only the decrease in ζ -potential and the concomitant decrease in the electrostatic repulsion between the phosphatidylserine particles to a certain threshold value were responsible for the precipitation then one would expect these quaternary ammonium compounds also to be precipitated. Although the short-chain alkylammonium ions and acetylcholine decrease the ζ -potential of phosphatidylserine particles in a similar way to Na^+ and K^+ , and atropine decreases the ζ -potential even more effectively, none of these compounds caused precipitation (see Tables 2 and 3). This surprising result throws light on another important property of these compounds that has been largely ignored, especially with regard to their physiological and pharmacological effects. The inability of these compounds to precipitate negatively charged lipid particles could arise from changes in the water structure of the hydration spheres associated with the colloidal particles and their counterions.

It is well established that many solutes are salted out by inorganic ions (Conway, 1966; Long & McDevit, 1952). Such salting-out can be regarded as competitive hydration and seems to be governed by electrostatic effects. It is not well understood in molecular terms. In contrast, non-electrostatic effects become important in the salting-in caused by alkylated ions. Such ions

are designated as hydrophobically hydrated in that they promote the formation of tetrahedrally structured water in their vicinity (Conway, 1966; Franks, 1968). The salting-in is presumed to arise from sharing of structure-enhanced regions by the salted-in and salting-in compounds (Conway, 1966). The distinction between the precipitation behaviour of the cations of groups 2 (salting-out) and groups 1 and 3 (salting-in) of Table 3 presumably arises because of the different overall effects of these cations on the water structure associated with the phosphatidylserine particles. Hydrophobically hydrated counterions promote water structure whereas electrostrictively hydrated inorganic counterions cause breakdown of the tetrahedral water structure by reorientation and immobilization of water molecules in their vicinity (Frank & Wen, 1957).

At present there is no information on the hydration of the biologically significant molecules of group 1 of Table 3. In this laboratory pulsed n.m.r. relaxation-time measurements have been made on acetylcholine chloride solutions that suggest that the effect of acetylcholine on water is similar to that of tetraethylammonium chloride (A. Darke & E. G. Finer, unpublished work).

The finding that acetylcholine and choline are hydrophobically hydrated may have importance in understanding the mode of action of acetylcholine at the cholinergic receptor. Electrostrictively and hydrophobically hydrated cations have different effects on the solubility of phosphatidylserine particles and on the tertiary structure of proteins in solution (von Hippel & Schleich, 1969). It therefore seems likely that similar effects will occur with lipoproteins and natural membranes, perhaps changing the permeability of the latter to ions. Such effects would probably tend to be rather non-specific and the stereospecificity observed in structure-activity relationships [e.g. (+)-acetyl- β -methylcholine and (-)-acetyl- β -methylcholine have effective molarities in their action on the post-ganglionic cholinergic receptor that differ 200-fold] still has to be accounted for. However, the particular hydration effects of choline bases as well as their conformation should be borne in mind when considering the mode of action of acetylcholine.

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