The structure and function of cholinergic synaptic vesicles

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On a recent visit to Marburg, a charming ancient university city in Hessen, the neighbouring state to the south of the one I live in—a city that boasts the oldest gothic church in Germany—I was pleased to learn that the name is still revered of a 19th century Hessian general practitioner who emigrated to London and there performed his pioneer and classical researches on the chemical composition of the brain (for a review see McIlwain, 1958). I refer of course to Dr. Thudichum for so his name was pronounced in Marburg. The name is unusual: perhaps it is *tue dich um, 'look about you', for *sich umtun nach etwas is to look about for something, and if this speculation (Drabkin, 1958) is correct it seems a particularly appropriate name for a research worker.

Dr. Thudichum emigrated from Germany to England to obtain better conditions for his work, so perhaps there is something appropriate in the award of a medal bearing his name to an English neurochemist who emigrated to Germany 10 years ago for much the same reason. We tend to think of the ‘brain-drain’ as a modern phenomenon, but it is as old as science itself: scientists have always worked for the Prince that gave them the best facilities for their work.

I regard the invitation to give this lecture as an honour not only to myself but to the many fine coworkers I have been and am privileged to have. Of these I would particularly mention Mike Dowdall, Herbert Zimmermann, Herbert Stadler, Veit Witzemann, Jan Suszkiw, Panos Giompres, Stephen Morris, Geoffrey Fox, John Walker, Theresa Jones, and 'last but not least' Gordon Dowe, my technician of 25 years standing, who has accompanied me in my wanderings, trained numerous visiting scientists in our methods and administers the laboratories in a wise and firm manner.

I think it is true to say that the discovery of the synaptosome in 1960 (Gray & Whittaker, 1962; Whittaker et al., 1964) greatly enlarged our idea of what subcellular fractionation methods can achieve and has proved of great use in understanding the molecular basis of synaptic function (Table 1). Synaptosomes are also taking on a new lease of life for ‘mitochondriacs’ interested in studying the interaction of the mitochondrion with its cytoplasmic environment (e.g. Scott & Nicholls, 1980). However, for those interested in a specific type of chemical transmission, in my case cholinergic transmission, it must be admitted that synaptosomes as prepared from mammalian brain have one serious disadvantage; they are heterogeneous with respect to transmitter type and probably less than 10% of them are of cholinergic origin.

The electric organ of Torpedo as a model cholinergic system

We therefore, some years ago, looked around for a richer source of cholinergic nerve terminals. Table 2 contrasts the acetylcholine content of two acetylcholine-rich tissues, squid optic lobes and the electric organ of *Torpedo, with two mammalian tissues, guinea-pig brain cortex and the purely

* Two Quinquennial Reports covering the work of the department during the period 1973-1983 are available on request.

Table 1. Synaptosomes

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Synaptosomal distribution is <em>prima facie</em> evidence of transmitter function</td>
<td>Are derived from many different types of nerve terminal, therefore are of mixed transmitter type</td>
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<tr>
<td>Retain most properties of the synapse in <em>vitro</em></td>
<td>May show only limited vesicle recycling</td>
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<tr>
<td>Are source for preparations of component organelles: synaptic vesicles, presynaptic plasma membranes, postsynaptic densities, presynaptic axoplasm</td>
<td></td>
</tr>
<tr>
<td>Are model cells for studying uptake mechanisms, mitochondrial function in <em>situ</em> etc.</td>
<td></td>
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cholinergic rat diaphragm. As can be seen, the optic lobes and electric organ are several orders of magnitude richer in acetylcholine than the mammalian tissues. Cephalopod brain is about twice as cholinergic as electric organ and was in fact the first nervous tissue in which acetylcholine was demonstrated to be present by unambiguous chemical methods (Bacq & Mazza, 1935), but it is none-the-less not purely cholinergic. Dowdall & Whittaker (1973) demonstrated that synaptosomes and synaptic vesicles could be isolated from it and showed that the synaptosomes had a high-affinity choline uptake system, the first demonstration of this system in cholinergic synaptosomes. However, in recent years we have tended to work more and more with a purely cholinergic source, the electric organ of *Torpedo*. The comparison with rat diaphragm is appropriate, since the cholinoreceptive cells of the organ, the electrocytes, develop embryologically from myotubes, a process known to 19th century investigators and recently thoroughly studied at the biochemical and electron microscopical level in my laboratory (Fox & Richardson, 1978, 1979). These cells are well known as extremely rich sources of the nicotinic acetylcholine receptor, the isolation and characterization of which is one of the success stories of modern neurochemistry. Veit Witzemann and Dieter Schmid of my Department, in collaboration with Bert Sakmann, have lately confirmed, using mRNA isolated from embryonic electric organ, the recent observation (Miledi et al., 1982) that the introduction of such mRNA into oocytes leads to the formation of functionally active and electrophysiologically detectable ion channels.

One hope is that the use of purely cholinergic tissue will lead to the identification (Walker et al., 1982) of cholinergic-specific surface antigens that can then lead to new histochemical methods and to new immunofluorescence techniques for separating out mammalian brain cholinergic synaptosomes from a mixed population (Richardson, 1983). Chol-1, as we call it (Richardson et al., 1982), is a surface antigen with the properties of a pentasialoganglioside which is specific for cholinergic terminals both central and peripheral in all species tested (Jones et al., 1981). This was first discovered in purified samples of electromotor presynaptic plasma membrane.

A vertical section (Fig. 1a) through a stack of electrocytes reveals that about 80% of the under-surface of each electrocyte is covered with nerve terminals. This highly polarized structure ensures that if the organ is activated by the synchronous discharge of the electric lobe the entirely normal postsynaptic potentials evoked by the release of acetylcholine sum in parallel and in series to form a sizeable electrical discharge: some 40V measured in air and quite enough to stun small prey whose presence in the environment is detected by the electroreceptors (ampullae of Lorenzini) sensitive to the action potentials generated by the prey's swimming muscles.

An area such as that ringed in the low-magnification electron micrograph shown in Fig. 1a when enlarged gives the more detailed fine structural information contained in Fig. 1b. It will be seen that the nerve terminals very much resemble those of muscle: they contain numerous synaptic vesicles which, however, are somewhat larger than those in normal motor nerve terminals, 90nm instead of 50nm in diameter.

**Isolation of cholinergic electromotor vesicles**

To isolate the vesicles in bulk for biochemical investigation we have resorted (Fig. 2) to a rather unorthodox method of tissue comminution: freezing in liquid nitrogen and pounding the now brittle tissue to a coarse powder in a ceramic mortar. Extraction with iso-osmotic sucrose, sucrrose/saline or other solutions gives, after removal of coarse and intermediate-sized particles, a vesicle-rich cytoplasmic extract which is then submitted to density-gradient fractionation in a zonal rotor (Whittaker et al., 1972). Fig. 3 shows the distribution of membrane-bound cholinesterase, acetylcholine and protein in such a gradient after a run. The position of acetylcholine in the gradient shows clearly

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**Table 2. Content of cholinergic synapses in different tissues**

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Nerve terminals (% w/v of tissue)</th>
<th>Acetylcholine (nmol/g)</th>
<th>Other transmitters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>Brain cortex</td>
<td>3</td>
<td>20</td>
<td>Yes</td>
</tr>
<tr>
<td>Rat</td>
<td>Diaphragm</td>
<td>0.001</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td><em>Torpedo</em></td>
<td>Electric organ</td>
<td>1</td>
<td>1000</td>
<td>No</td>
</tr>
<tr>
<td>Squid</td>
<td>Optic lobes</td>
<td>5</td>
<td>2000</td>
<td>Yes</td>
</tr>
</tbody>
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Fig. 1. *Vertical section through a stack of electrocytes*

In (a) portions of four electrocytes are shown. Note beaded appearance of innervated, ventral (v) surface and fuzzy appearance of non-innervated, dorsal (D) surface, the latter due to numerous invaginations. In (b) a portion of the ventral surface, similar to that ringed in (a), is enlarged showing invaginations of the postsynaptic membrane (V) and presynaptic electromotor nerve terminals filled with synaptic vesicles which are the beads seen in (a). Scale bars: (a) 10μm, (b) 1μm.
Freeze in liquid nitrogen
Crush
Extract
Centrifuge
Pellet (discard)

Fig. 2. Flow diagram for bulk isolation of synaptic vesicles
The supernatant obtained after centrifuging the extract of frozen and crushed tissue at 3.10^5 g" min is fractionated in a density gradient on a zonal rotor directly (Whittaker et al., 1972; Ohsawa et al., 1979) or after a preliminary concentration of the vesicles in a step gradient containing EGTA (Tashiro & Stadler, 1978). SP, Soluble protein; VP, synaptic vesicles; MP, small membrane fragments.

Fig. 3. Distribution of protein (O), acetylcholine (□) and membrane-bound cholinesterase (△) in the zonal gradient (………), shows sucrose concentration, forming peaks of soluble protein (SP), synaptic vesicles (marker: bound acetylcholine) (VP) and small membrane fragments (marker: bound cholinesterase) (MP)

that it is bound to particles and not in the free state. Its co-existence with considerable amounts of solubilized and membrane-bound cholinesterase shows that it is sequestered within these particles and not accessible from the suspension medium. However, methods that disrupt lipoprotein membranes, freezing and thawing, hypo-osmotic shock or detergent treatment, release acetylcholine and allow its destruction by the circumambient cholinesterase. Dowdall et al. (1974) showed that ATP is a constituent of these vesicles, as it is of chromaffin granules, and is subject to the same considerations.

Structure of synaptic vesicles
Morphology. Fig. 4, an electron micrograph of the particulate material in the acetylcholine peak, shows what these particles that sequester acetylcholine look like. They are instantly recognizable as the synaptic vesicles we see in intact tissue (Fig. 1b). Analysis shows (Ohsawa et al., 1979) that there are about 200000 molecules of acetylcholine and 30000 molecules of ATP in the vesicles, corresponding to a combined concentration of close to 1 M. N.m.r. studies by Stadler & Fültdner (1980, 1981) and Fültdner & Stadler (1982) have shown that both these components are in a free state. The combined osmotic pressure they exert corresponds to the 800–900 mosm of Torpedo body fluids. The limiting concentration of acetylcholine in vesicles that have not been subject to excessive depletion of membrane protein by treatment with chelating agents is about 6–7 nmol/mg of protein, consistent with the values I have already cited and the stoichiometry of the vesicle membrane. Work by Weiler et al. (1982) shows that 80% or more of tissue acetylcholine is vesicular.

Protein composition. Extensive work by Tashiro & Stadler (1978) and Stadler & Tashiro (1979) on the protein composition of the purified vesicles has led to the recognition of five to six well-defined major constituents. As shown by a com-
parison of the two-dimensional gel pattern obtained from vesicles with that obtained from electromotor presynaptic plasma membranes (Fig. 5), there is only one protein in common, no. 8: actin, a special type of actin (Zechel & Stadler, 1982). This confirms a view I have long held (Whittaker, 1966), namely that vesicle exocytosis does not involve total fusion of the vesicle membrane with the plasma membrane and loss of its identity, but rather that vesicle exocytosis is a transient phenomenon and is followed by complete vesicle membrane retrieval and reconstitution of a functional vesicle. Axoplasmic transport of vesicle constituents has been detected but is insufficient to supply vesicles in sufficient number on a 'throw-away' or 'no re-use' basis.

The other proteins appearing in the two-dimensional vesicle protein gel (Fig. 5a) can be identified, at least tentatively, as follows: 1 and 2 are components of a Ca$^{2+}$, Mg$^{2+}$-activated ATPase; 11 has been identified with great certainty as the result of the work of Lee & Witzemann (1983) as the ATP/ADP translocase of the vesicle membrane; component 13 may well be the acetylcholine translocase. A minor, but functionally important, component is a proton-translocating ATPase which was present in insufficient amount to show up in this gel. We know from n.m.r. (Füldner & Stadler, 1982) and radioactive methylamine uptake studies (H. Stadler, unpublished work) that the inside of the vesicle is 1.5pH units more acid than the surrounding cytoplasm. Harlos & Stadler (1983) have demonstrated by means of methylene chloride extraction the presence of a component of $M_r$ 53000 very similar to, but not identical with, the $F_1$ component of the $F_0/F_1$ mitochondrial proton-translocating ATPase.

Fig. 6 shows a gel-separation of this protein after $^{125}$I-labelling: its presence in the vesicular extract cannot be explained by mitochondrial contamination for as shown in this Figure, the mitochondrial extract shows a more complex pattern than the vesicular. Another difference is that this ATPase is not oligomycin-sensitive.

**Vesicle-specific proteoglycan.** Another protein component which does not readily show up in Coomassie Blue-stained gels of vesicle proteins is a proteoglycan of the heparan sulphate type which is the most antigenic of vesicle constituents and can also be labelled *in vivo* with $^{35}$S injected into the electric lobes, the paired lobes in the brain of *Torpedo* which contain the cells of origin of the electromotor nerves (Stadler & Dowe, 1982). Our immunohistochemical studies show that this proteoglycan is vesicle-specific. Unfortunately cross-reactivity does not extend to all cholinergic vesicles, at least with the titres we have available. However, the sera can be used to semiquantify the distribution of the proteoglycan and we thus have a vesicle marker independent of acetylcholine and ATP and of the vesicles' structural integrity. This serum can also be used immunohistochemically to trace the synthesis, transport and fate of vesicles, as has been elegantly shown by Jones et al. (1982a,b). Both immunohistochemical studies and immunoochemical studies using $^{35}$S-labelled vesicles show that the antigen is inside intact vesicles and only becomes accessible when vesicle structure is disrupted, e.g. by detergents or osmotic shock. There is one exception: in stimulated nerve terminals *in situ*
the antigen becomes exposed during stimulus-induced exocytosis and is re-internalized during recovery at rest.

Vesicle-specific proteoglycan has a somewhat tangled history. The heparan sulphate-like glycosaminoglycan residues are readily split off and are released if vesicles are disrupted, e.g. by prolonged dialysis against water (Stadler & Whittaker, 1978). The glucosaminoglycan readily binds ATP and amino acids present in crude vesicle preparations. This is also a previously unsuspected property of heparin from mast-cell granules. Such amino acids together with traces of contamination protein confused us initially into thinking that it was a small molecular mass protein that was released on dialysis instead of a glucosaminoglycan: we gave it the name vesiculin (Whittaker et al., 1974). Thus our vesicle-specific proteoglycan is in fact a vesiculin–protein conjugate. However, we now prefer the longer, but more accurate, term 'cholinergic synaptic vesicle-specific proteoglycan'.

Figs. 7 to 9 show some histochemical studies performed with our anti-proteoglycan serum by Jones et al. (1982a). Except for electric organ, Paraplast embedding and (for axons) Triton treatment of the section was used to expose the antigen.

Fig. 7a shows the punctuate distribution of the antigen in the cell body of the electromotor neuron; Fig. 7b is a phase-contrast micrograph of the same structure. The punctate distribution suggests that the proteoglycan is already packaged in vesicles and that these have accumulated in the axon hillock awaiting transport.

Fig. 8 shows the accumulation of proteoglycan above a ligature in the electromotor axon. We know that acetylcholine and ATP similarly accumulate (Davies, 1978). Much
Fig. 8. Indirect immunofluorescence histochemistry with an antiserum to vesicle-specific proteoglycan (Walker et al., 1982) of Paraplast section of axon of Torpedo marmorata. A ligature (arrows) was placed around the nerve 5 days before histochemistry. The section was additionally pretreated with Triton X-100 to improve penetration of antiserum. Magnification × 300. EO, Electric organ. From Jones et al. (1982a).

Fig. 9. Indirect immunofluorescence histochemistry with an antiserum specific to vesicle-specific proteoglycan (Walker et al., 1982) of electric organ of Torpedo marmorata. (a) Cryostat section. (b) Tissue stimulated at 5 Hz for 10 min before cryostat sectioning. Note that only the ventral surface of electrocyte responds and that only after stimulation. Scale bar: 10 μm. From Jones et al. (1982a).
more needs to be done on the axonal transport of cholinergic synaptic vesicles, but we now have the tools to do it with.

Unlike those of Figs. 7 and 8, the sections shown in Fig. 9 were not treated with Paraplast and Triton. The micrograph in Fig. 9a shows the rather faint background staining obtained with resting tissue. The micrograph in Fig. 9b shows the intensification of the staining on the ventral surface of the electrocyte following stimulus-induced transmitter release. This intensification of staining is reversible: if the block is allowed to recover, the staining reaction fades again; if restimulated, it returns (Jones et al., 1982b).

Fig. 10 shows the interpretation we put on this result. Fig. 10a shows that only vesicles cut open by the cryostat sectioning will have accessible antigen (shown as black spots). Such nerve endings stain feebly when not Tritonized. On stimulation many more vesicle interiors become transiently

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Fig. 10. Explanation proposed for increase in fluorescence of ventral surface of electrocyte seen in Fig. 9 on stimulation

Black spots represent vesicle-specific proteoglycan made accessible by sectioning (a) or sectioning plus stimulus-evoked vesicle exocytosis (b). The proteoglycan is reinternalized on recovery from stimulation (Jones et al., 1982b). S, Stimulation; R, recovery.

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Fig. 11. Section of electromotor neuron perikaryon

Section shows Golgi membranes (G), nascent vesicles (V), mitochondria (M), pigment granules (Pg) and polysomes (Po). Scale bar: 0.2 μm.
accessible to antiserum as a result of exocytosis, so that staining intensity increases.

The function of the proteoglycan is not known but it may serve to provide negative charges to balance those positive charges of acetylcholine, calcium and magnesium not balanced by the vesicular ATP. It may also help the vesicle membrane to conserve its identity through cycles of exo- and endocytosis.

Cell-free synthesis of vesicular proteins. Electromotor nerve cell perikarya have to maintain about 30 times their volume of synapse. This presupposes an intense synthetic activity and this is borne out by the appearance of the perikaryal cytoplasm, loaded with Golgi membranes and polysomes (Fig. 11). It is not surprising that the mRNA isolated from these cells is highly active in a cell-free translation system, as shown by Schmid et al. (1982): some of the products can be clearly identified as synaptic proteins after two-dimensional gel electrophoresis (Fig. 12) as shown by the spots numbered to correspond with those of Fig. 5a, and other preterminal synaptic components indicated by arrows. Schmid has now succeeded in cloning the complementary DNA corresponding to this mRNA and by using suitable probes he hopes to identify the portions of the genome coding for cholinergic function.

Water content of synaptic vesicles. Another approach we have made for the understanding of vesicle structure has been to measure the various water contents of the vesicle. Giompres et al. (1981a) determined water spaces by a simple technique. The densities vesicles assume when they are centrifuged to isopycnic equilibrium in iso-osmotic density gradients made up in water and in a penetrating denser solvent are compared. The space occupied by the solvent within the vesicle is then $\Delta \rho / \Delta \rho$ where $\Delta \rho$ is the difference in density of the particles in the two gradients and $\Delta \rho$ is the difference in density of the solvents. Glycerol measures the osmotically active, free water, dimethyl-sulphoxide measures in addition the water bound to hydrated solutes like acetylcholine and ATP and deuterium oxide measures both these waters and in addition the water of the lipoprotein membrane.

Summary of structural information. Fig. 13 summarizes all the findings I have described which have gradually been amassed during the last 10 years of research. Careful measurements by Ohsawa et al. (1979) have shown that there is just enough lipid per vesicle to make a 4 nm thick bilayer round an 82 nm diameter core. There is just enough protein in the membrane to confer upon it the measured density of 1.13 g/ml. Water-space measurements with deuterium oxide show that the membrane is highly hydrated, about 30% of it is water. We have seen that the membrane contains a Ca$^{++}$, Mg$^{++}$-activated ATPase, a few copies of a proton-translocating oligomycin-insensitive ATPase of the F$_{1}$/F$_{0}$ type, translocases for acetylcholine and ATP, and actin; also that the internal pH is acidic. A proteoglycan projects into the core and helps to neutralize the positive ions present: acetylcholine, H$^{+}$, Mg$^{++}$ and par-

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**Fig. 12.** Tracing of radioautograph of translation products obtained by addition of poly(A) mRNA isolated from electromotor neuron perikarya to a cell-free rabbit reticulo-cyte translation system, and separated by two-dimensional sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

R is the sole product obtained with the unfortified system. The open profiles trace the radioactive translation products; the black spots, Coomassie-Blue-positive components of a synaptosome preparation derived from electric organ. The numbered spots correspond to those shown in Fig. 6. The arrows mark non-vesicular synapto-somal components. Vesicular components 1 and 2 are not synthesized; 8, 11 and 13 are and apparently do not require post-translational modification.
Recycling of synaptic vesicles

We may now ask: what happens to the vesicle when the nerve terminals are stimulated? It occurred to me some years ago that by analogy with the successful manipulation of the density of lysosomes by inducing rat liver to take up light or dense substances (Thines-Sempoux, 1967), it should also be possible to modify the density of that subpopulation of vesicles undergoing cycles of stimulus-induced exo- and endocytosis by introducing a dense material such as dextran from the extracellular space during the brief time the vesicle interior is exposed to it. Such loaded vesicles should then be separable from the non-recycling, reserve population.

Herbert Zimmermann undertook this work and his first step was to devise a perfused electric organ preparation so that dextran and other substances, including radioactive precursors of acetylcholine, could be brought to the vicinity of the active electromotor nerve terminals in appropriately high concentration (Zimmermann & Denston, 1977a,b). Fig. 14a shows a perfused preparation; in other experiments, the territories of nerves I and IV may be excised and two cannulae inserted into the blood sinuses accompanying each nerve. Either way one fish gives four innervated blocks. The blocks may be stimulated through the nerves, or by field stimulation, or they may be used to study the recovery of tissue stimulated in vivo. Jan Suszkiw introduced the use of small blocks or prisms suspended in Ringer solution shown in Fig. 14b which are convenient for some purposes (Suszkiw et al., 1978; Suszkiw, 1980).

Morphological studies in situ. In situ, two changes are observed to occur in vesicles: they move to the presynaptic plasma membrane, if dextran is present they acquire it, and the vesicles close to the membrane, and containing dextran, become about 25% smaller in size than the unlabelled ones (Fig. 15). In time 80% of the vesicle profiles, shown by the dashed line in Fig. 16, belong to the smaller size range and
Fig. 16. Distribution of vesicle profile diameters in control, resting block (continuous line) and in a block after stimulation at 0.1 Hz for 5h during perfusion with dextran-containing medium (dashed lines)

Note bimodal distribution of diameters in latter with original vesicle population represented by a prominent shoulder. The filled-in blocks give the profile diameter distribution of those vesicles in the stimulated block that have acquired dextran particles. Only the smaller vesicles have acquired dextran. Distributions have been normalized to 100%, but there was little change in vesicle content. Numbers in parentheses give numbers of profiles measured (Zimmermann & Denston, 1977a).

only a shoulder indicates the presence of a small reserve population without dextran with a mean profile diameter similar to that of the original population. The distribution of the diameters of dextran-containing vesicles is shown by the filled-in blocks. These changes are reversible.

**Metabolic heterogeneity.** Fig. 17 compares the distribution of acetylcholine in a zonal density gradient prepared from unstimulated tissue (Fig. 17a) and from stimulated tissue (Fig. 17b).

It can be seen that as before, acetylcholine in gradients derived from unstimulated tissue forms a single vesicle peak, and that in gradients derived from stimulated tissue it is present in lesser amounts and is bimodally distributed. Examination of the distribution of vesicle profile diameters (Fig. 17 insert) shows that the mean diameter of vesicles from the dense peak (distribution shown by dashed line) is about 25% less than that from the lighter vesicle peak (continuous line); moreover, if dextran is present dextran-containing vesicles (filled-in blocks) are found exclusively in the dense peak, and then after sufficient stimulation up to 80% of profiles may show the label. It is clear that the smaller and larger vesicles observed in situ have been isolated and separated from each other.

The functional significance of the two populations is further indicated by experiments in which, as here, a radioactive precursor, usually [3H]acetate is perfused. Fig. 17a shows that while the vesicles in resting tissue do take up some radioactive acetate, the labelling of the vesicle compartment is not impressive. In vesicles derived from stimulated tissue, the situation is far different: to be sure, the light vesicle peak, corresponding to the vesicle present in resting tissue, is little labelled, but the new peak of smaller, denser, recycling vesicles is highly labelled. Clearly, for vesicles to take up newly synthesized acetylcholine from the cytoplasm, they must first be emptied of pre-existing transmitter by undergoing recycling. As shown by the arrow in Fig. 17a, when once this fraction of vesicles has been recognized, small amounts of it can be identified in the resting tissue as a small highly labelled shoulder on the descending limb of the unimodal vesicle peak. It is known that electric organ, like muscle, is never completely silent; miniature potentials can be recorded in resting tissue, indicating a low level of spontaneous vesicle recycling in the absence of applied stimuli. I should also emphasize that the separation of the two classes of vesicle occurs in the absence of dextran or other dense solute.

Fig. 17. Distribution, in zonal gradients, of the cytoplasmic marker lactate dehydrogenase (LDH) (■), the vesicle marker acetylcholine (ACh) (△) and radioactive acetylcholine (○) after fractioning cytoplasmic extracts from (a) unstimulated and (b) stimulated blocks that had been perfused with medium containing dextran and [3H]acetate

The asterisk marks the location of a small fraction containing cytoplasmic inclusions (synaptosomes?) and the arrow a small shoulder of highly labelled vesicular acetylcholine present in unstimulated blocks corresponding to the VP1 fraction in stimulated blocks. Insert: distribution of vesicle profile diameters in VP1 and VP2: the filled-in blocks give that of vesicles in VP2 containing dextran particles. No dextran-containing profiles were present in VP1 (based on results of Zimmermann & Denston, 1977b).
A prediction can now be made: if after a loading stimulus, a second short stimulus is given, release will take place preferentially from the recycling population that has been established and what is released will reflect the content of the recycling population and not that of the other compartments of transmitter: the cytoplasm and the reserve vesicles.

Yunus Luqmani verified this prediction using tritiated homocholine (Luqmani et al., 1980). This substance is taken up by electromotor nerve terminals and is partially converted to acetylhomocholine by the choline acetyltransferase of the cytoplasm. Acetylhomocholine is a better substrate for vesicle uptake than homocholine so that the acetylhomocholine to homocholine ratio in the recycling vesicles is about 10 times greater than it is in the cytoplasm. The reserve population is essentially unlabelled. On restimulation the ratio in which these two false transmitters, for such we may call them, are released is equal to that found in the recycling vesicle fraction and not in the cytoplasm.

Fig. 18a shows that the tritiated homocholine and acetylhomocholine go selectively into the recycling (VP, peak). On restimulation (Fig. 18b) homocholine and its ester are released. On restimulation (Fig. 18c) homocholine and its acetate ester appear in the same ratio in the perfusate as they do in the VP, vesicles after or before stimulation, whereas the ratio of the two transmitters in total tissue or cytoplasm is quite different.

Water and solute content of recycling vesicles. As we have

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**Fig. 18.** Evidence that false transmitters preferentially enter the VP, vesicle population and are released from it

(a) Tritiated homocholine and acetylhomocholine (----) go into the VP, peak identified by the distribution of 14C-labelled acetylcholine (•••••••). This peak and VP, are defined by the distribution of the alternative vesicle marker ATP (Dowdall et al., 1974) (histogram). The blocks from which the vesicles were isolated had been perfused in closed circuit with [3H]homocholine and [14C]choline while being stimulated at 0.1 Hz for 1 h and then allowed to recover for 5 h. (b) Release of homocholine and acetylhomocholine into perfusate on restimulation (10Hz for 10min) of a block loaded as in (a) but perfused with [3H]homocholine alone. The recovery period included an open-circuit ‘wash-out’ perfusion with medium containing 100mM-paraxon during the last 3h of recovery. (c) Ratio of homocholine to acetylhomocholine in (R) perfusate during release stimulus, compared with that in VP, vesicle (V), cytoplasm (C) or total tissue (T) samples from blocks that had (subscript s) or had not (subscript c) received the release stimulus (Luqmani et al., 1980). R refers to stimulated release, i.e. total release corrected for base-line release.
seen, separation of the two classes of vesicle occurs even in the absence of dense solutes such as dextran. Why should recycling vesicles be smaller and denser than reserve vesicles? Biophysical studies made by Giompres et al. (1981a,b) have convinced us that the difference in size and density of the two populations can be quite precisely accounted for by an osmotically driven change in water content. When water is lost the vesicle shrinks and becomes denser. This is attendant upon a lower content, in the recycling vesicles, of osmotically active small molecules, mainly acetylcholine and ATP. This in turn implies a two-stage refilling process: a rapid uptake of acetylcholine and ATP during recycling and a slower uptake of acetylcholine, ATP and water during recovery after stimulation. This second uptake process allows the recycled population to re-acquire the physical properties of the reserve population. From Fig. 19 it can be seen that the density of the recycling vesicles falls during 24h from its value (1.065 g/ml) immediately after the end of stimulation to the value characteristic of vesicles in resting tissue (1.055 g/ml), and that this fall occurs pari passu with a rise in the vesicle's content of water and solutes (acetylcholine, ATP).

One simple way of detecting differences in the content of osmotically active molecules is to construct osmotic fragility curves. Vesicles behave like osmometers over a certain range, varying their density and water content reversibly with changes in osmotic pressure. If the osmotic pressure is reduced too far, the inflow of water becomes too much for the vesicle membrane: it bursts and there is a loss of acetylcholine and ATP. The curves shown in Fig. 20 (Giompres & Whittaker, 1984) are the osmotic fragility curves for the two populations of vesicles. They show that the dense, recycling vesicles (fraction VP2) are more resistant to hypo-osmotic conditions than the reserve vesicles (fraction VP1), consistent with a lower content of osmotically active molecules per vesicle.

Another way that this has been shown is by measuring the ratio of acetylcholine to the vesicle membrane marker vesicle-specific proteoglycan (V. P. Whittaker, J. H. Walker & G. H. C. Dow, unpublished work). The proteoglycan copurifies with the single peak of vesicles from unstimulated tissue in a zonal gradient. In a gradient of material derived from stimulated tissue the proteoglycan, like acetylcholine, is distributed bimodally: however, the acetylcholine to proteoglycan ratio is considerably lower in the recycling vesicle fraction VP2 than in the reserve VP1 population (Fig. 21).

A de Duve plot of the gradient is shown in Fig. 22. The mean relative specific concentration of acetylcholine relative to proteoglycan in all the VP, fractions is ± 0.53 ± 0.03.

I mentioned earlier that we believe that the fractional glycerol space ($v_g$) at 800 mosmol measures the osmotically active water space. Fig. 23 shows that, indeed, the fractional glycerol space at other osmotic pressures, $v_g$, diminishes for both vesicle populations pari passu with the increasing density induced by osmotic dehydration (P. E. Giompres & V. P. Whittaker, unpublished work). With a linear density scale as in Fig. 23, the osmotic pressure scales are not identical for the two populations.

It is instructive to compare $v_g$ measurements with the fractional water space $v_w$ calculated from the amount of water which has to be removed from an otherwise unchanged vesicle of density $\rho$ to generate the observed change to density $\rho'$. This can be calculated as follows. Since

$$\rho' = \frac{\rho V - w}{V - w}$$

$$w = V\frac{\rho' - \rho}{\rho' - 1} = V_q$$

where $V$ is vesicle volume at density $\rho$ and $v_w = V_w'/V' = (V_w - w)/(V - w) = \frac{V_w - q}{1 - q}$

where $V_w$ and $V_0$ are the water spaces at densities $\rho$ and $\rho'$, $V'$ is vesicle volume at density $\rho'$ and $v_w$ is the fractional water space at density $\rho$.

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**Fig. 19.** Recovery at rest by VP2 vesicles of biophysical and biochemical characteristics of VP1 vesicles.

- (□) Density ($\rho$);
- (■) free water content (glycerol space, $v_g$);
- (○) acetylcholine;
- (●) radioactive acetylcholine;
- (▲) ATP (Giompres et al., 1981b).

**Fig. 20.** Osmotic fragility curves for (△) VP1 and (▲) VP2 vesicles (Giompres & Whittaker, 1984)
These estimates are plotted in Fig. 23 and it can be seen that they do not agree with the \( v_o \) measurements. Why should this be? Why should \( v_o \) fall below \( v'_o \)? A possible explanation is that when a vesicle membrane collapses round an osmotically dehydrated core some of the remaining water enters a vesicle compartment inaccessible to glycerol. The most likely compartment is the membrane.

Fig. 24a shows the swelling of the membrane that must occur to accommodate the water that has not been removed by osmotic dehydration but which has become inaccessible to glycerol. One can consider two models: one in which the spherical shape of the vesicle is conserved and another, the 'squashed-in-tennis-ball' or constant area model.

Fig. 24b also shows the increase in membrane thickness which must occur on the two models. It is clear that after a certain water loss, the membrane swelling on the constant area model is considerably less than on the spherical model. We therefore believe that osmotic collapse is accompanied by a change in configuration of the vesicle as has been observed directly with chromaffin granules. These studies show the descriptive power of simple biophysical methods.

We (V. P. Whittaker, G. H. C. Dowe, I. S. Roed & J. Lisziewicz, unpublished work) are now studying the
kinetics of the recovery process. This is sensitive to the metabolic state of the tissue. Fig. 25a shows superimposed zonal runs of vesicles extracted from perfused blocks derived from organs stimulated \textit{in vivo} at 1 Hz for 1 h and normalized to 100%. Eserinization was used to preserve the cytoplasmic pool. When results are expressed as 100\% of the 4 h total (Fig. 25b) one can see that the proportion of vesicles in the recycling pool rises rapidly at first, and then plateaus, while the reserve population, small at first, recovers later at the expense of a continued increase in the VP₂ population. The cytoplasmic fraction remains a small constant pool throughout. We hope that experiments of this kind, conducted under different conditions of stimulation, will define the kinetics of the recovery process.

Recent work by Denés Ágoston (D. Ágoston, unpublished work) shows that the 'VP₁–VP₂' phenomenon is not confined to \textit{Torpedo} but that cholinergic vesicles from the myenteric plexus of guinea pig also become bimodal in density and specific isotope concentration on stimulation (Fig. 26). The density differences are smaller but this can be accounted for by the smaller size of the vesicles. Similar phenomena have also been observed with brain vesicles (Barker \textit{et al.}, 1972).

Some of you may wonder where we get our \textit{Torpedos} from. The answer is a marine biological station in the small French seaside resort of Arcachon just south of Bordeaux (see Fig. 27). We rent a couple of laboratories there as an outstation for tissue culture and cloning work. The map in Fig. 27 also shows that both my laboratories, as befits a loyal Englishman working on the continent, are in territories which were formerly united with the English Crown: Hanover for 123 years between 1714 and 1837 and Aquitaine which from 1137 to 1451 was English territory and in 1154 became part of the great Plantagenet empire of Henry II which stretched from the Shetland Islands to the Pyrenees.

In conclusion I would like to bring to the Neurochemical Group of the U.K. Biochemical Society the fraternal greetings of Studiengruppe Neurochemie der Gesellschaft für Biologische Chemie founded in 1976 on the model of the Neurochemical Group and of which I had the honour to be elected the first Sprecher.
Fig. 25. Kinetics of vesicle recovery after stimulation

(a) Superimposed zonal runs of extracts from perfused, eserinized blocks derived from organs stimulated in vitro at 1 Hz for 1 h and allowed to recover for 0.5–4 h. Each curve is normalized to 100% and the acetylcholine content plotted as a function of the gradient volume around the peak at refractive index (RI) 1.3550. (b) Acetylcholine of VP1, VP2 and SP (cytoplasmic fraction stabilized by eserine) plotted, with correction for varying recoveries, as % of parent (S12) fraction at 4 h. The dotted line relates to whole tissue and its coincidence with that for the parent S12 extracts shows that these are comparable samples of the total tissue.

Fig. 26. Distribution of vesicular endogenous (d0) and deuterated (d4) acetylcholine in zonal density gradients of extracts of guinea-pig myenteric plexus-longitudinal muscle labelled with d4 choline without (a) or with (b) stimulation at 1 Hz for 10 min.

Note increased bimodality of vesicle peak from the stimulated relative to that of unstimulated preparation and greater isotope incorporation in denser (VP2) fraction.
Fig. 27. Map of Western Europe showing location of Göttingen and Arcachon and extent of Hanoverian and French possessions of the English crown.