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Cholinergic synaptic vesicles are metabolically and biophysically heterogeneous even in resting terminals

V.P. Whittaker

Arbeitsgruppe Neurochemie, Max-Planck-Institut für Biophysikalische Chemie, Göttingen (F.R.G.)

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The metabolic heterogeneity of synaptic vesicles in the cholinergic nerve terminals of the electromotor neurons of *Torpedo marmorata* has been studied in resting tissue by evaluating the molecular acetylcholine content (MAC) of synaptic vesicles after extraction from frozen and crushed tissue and high-resolution centrifugal density gradient separation in a zonal rotor. Although vesicular acetylcholine was distributed in the gradient as a single, more or less symmetrical peak, 3 subpopulations of synaptic vesicles could be identified: a small, relatively light subpopulation of low MAC on the ascending limb of the acetylcholine peak, designated V_0, a main population of fully charged vesicles designated V_1, and a small, denser subpopulation also of low MAC on the descending limb of the acetylcholine peak, designated V_2. The mean proportions and MACs of the 3 pools were: V_0, 13%, 58,000; V_1, 53%, 246,000; V_2, 34%, 79,000. When tritiated acetate was perfused through excised blocks of electric organ for 1–2 h before vesicle isolation, the specific radioactivity of the acetylcholine in the V_0 and V_2 pools was 10–30 times higher than in the V_1 pool. This suggests that both the V_0 and V_2 pools are not generated by the isolation procedure but are present in the intact endings and are functionally active. On the basis of their density and uptake of newly synthesized acetylcholine, the V_0 and V_2 pools were identified with the previously described VP_0 pool of axonal vesicles and the VP_2 pool of recycling vesicles in stimulated nerve terminals respectively. Since stimulation of electromotor nerve terminals is known to generate large proportions of VP_2 vesicles, variations in the proportion of V_2 vesicles in unstimulated tissue are attributed to varying amounts of adventitious stimulation of the tissue during dissection and perfusion.

INTRODUCTION

Historically, a difficulty for the synaptic vesicle theory of evoked transmitter release[36] was the observation that, in synapses in which the transmitter pools had been labelled with radioactive precursors, the specific activity of released transmitter was usually considerably higher than that of the main fraction of isolated vesicles[9]. This paradox was resolved when it was found that synaptic vesicles are metabolically heterogeneous and that those vesicles actively taking up and releasing newly synthesized highly radioactive transmitter constituted a recycling subpopulation which could be separated from fully-charged, metabolically inert reserve vesicles by density-gradient centrifuging[4,6,8,41] or particle exclusion chromatography[17]. This has been shown for mammalian brain[8] and myenteric plexus[5] and *Torpedo* electromotor nerve terminals[6,41]. The two vesicle pools have been designated VP_1 (reserve) and VP_2 (recycling).

Although stimulation markedly increases the proportion of recycling vesicles in the total population of synaptic vesicles, recycling vesicles are also present in resting' tissue[40,41]. Thus, when synaptic vesicles are isolated from unstimulated blocks of *Torpedo* electric tissue perfused with radioactive choline or acetate and centrifugally separated on a density gradient, the single peak of vesicular acetylcholine often shows a more or less pronounced shoulder on its dense side and, although there has been relatively little uptake of radioactive transmitter, that in the shoulder region has a much higher specific radioactivity than the main peak. Since this is the region of the gradient from which recycling vesicles are recovered in preparations made from stimulated tissue, it seems reasonable to conclude that resting tissue too contains a pool of recycling vesicles. This may arise as a result of spontaneous release, as evinced by the occurrence of miniature postsynaptic potentials[11,12,30] or adventitious stimulation during the removal of the tissue.

Evidence has recently been obtained, by pulse-labelling of vesicular proteoglycan, for the presence, in resting electromotor nerve terminals, of a pool of recently transported 'empty' synaptic vesicles (designated VP_0) that are less dense than the main, transmitter-rich population, but equal in density to vesicles isolated from the electromotor axons[21]. The existence of such vesicles, and of VP_2-type vesicles, in resting electromotor terminals is also suggested by the tendency of stable synaptic vesicle markers such as vesicular proteoglycan[32] and the

Correspndence: V.P. Whittaker, Arbeitsgruppe Neurochemie, Max-Planck-Institut für Biophysikalische Chemie, Göttingen, F.R.G.

putative vesicular ATP transporter[23,31,32] to form a broader peak than acetylcholine in the density gradient.

The purpose of the present work was to analyse more fully the various pools of synaptic vesicles present in the resting cholinergic synapse, by making further use of this now well-established and highly reproducible technique of centrifugal density gradient separation in a zonal rotor, of cytoplasmic extracts of electric tissue, well known as a rich source of the purely cholinergic nerve terminals of electromotor neurons[6,7,10,25,27,34,38,42]. The distribution of synaptic vesicles in the gradients was compared with that of acetylcholine and the various vesicle pools were identified by their mean molecular acetylcholine contents.

MATERIALS AND METHODS

Isolation and fractionation of synaptic vesicles

The general procedure used here has already been fully described[6]. Briefly, live specimens of *Torpedo marmorata* obtained from the Station Biologique d'Arcachon were kept in tanks of circulated, aerated and filtered artificial sea water at 15–17 °C until used. Electric organs were removed from fish under Tricaine anaesthesia induced by immersion in sea water containing 0.05% Tricaine methanesulphonate (Sandoz MS 222 supplied by Sigma, Deisenhofen, FRG) for 15–20 min by which time responses to tail-pinching had ceased. Suitably sized blocks of tissue (mean weight 60 ± 7 (4) g; here and elsewhere means are given ± S.E.M. with the number of observations in parentheses) were frozen, either immediately or after perfusion at 20 °C in a closed circuit for 1–2 h with *Torpedo* Ringer's solution (composition in mM: NaCl 280, KCl 3.5, $MgCl_2$ 1.2, $CaCl_2$ 3.4, glucose 10, urea 300, sucrose 100, $NaHCO_3$ 5.0, NaH_2PO_4 1.2, adjusted to pH 7.3) to which [^{3}H]acetate (2 μCi·ml^{-1} of specific radioactivity 100 μCi·mmol^{-1} from New England Nuclear, Dreieich, FRG) has been added, followed by 15 min open circuit perfusion with radioacetate-free medium. Weight increases in the perfused blocks of ca. 30% indicated satisfactory perfusion.

Cytoplasmic extracts were prepared from the frozen tissue by crushing it and extracting the resultant coarse powder while still frozen with a small volume of 0.4 M NaCl approximately equal to the tissue weight; they were then centrifuged at 10,000 rpm for 30 min to remove larger particles; the supernatant so obtained was sampled [3 ± 1 (4) ml] and the remainder [34 ± 2 (4) ml equivalent to 54 ± 6 (4) g of tissue] applied to a sucrose-NaCl density gradient formed[6] in a Beckman Ti-60 330-ml capacity zonal rotor. After centrifuging at 50,000 rpm for 3 h, the gradient was pumped out and collected in 5 ml fractions. The zonal centrifugation procedure has been fully described[6,38] and was carried out in the cold (4 °C). The gradient became hyperosomotic when its refractive index (RI) exceeded 1.3700.

Analytical methods

Acetylcholine was assayed on small strips of the dorsal muscle of the leech as previously described[37] after having been released from particulate material in cytoplasmic extracts and density gradient fractions and stabilized by bringing samples of the fractions to pH 4.0. Samples were stored at −20 °C until assayed.

ATP was determined in deproteinized supernatants of samples of subcellular fractions by a modification[10] of the luciferin-luciferase bioluminescence method[33]. The distribution of ATP in the gradients closely followed acetylcholine and its assay was done mainly as a rapid means for locating the fractions containing synaptic vesicles before assaying for acetylcholine and other markers.

Protein was determined by the bicinchonitric acid method[29] using a reagent kit supplied by the Pierce Chemical Co (Rockford IL, USA).

Sucrose in density gradients was determined by measuring the RI in an Abbé refractometer. To facilitate comparisons between different zonal runs in which gradient profiles may differ slightly, analyses of gradient components were plotted as a function of RI, not of fraction number. From the RI, the sucrose concentration and density of fractions may be deduced from tables. In the main part of the gradient the RI rose at the rate of 0.0006–0.0008 units per 5-ml fraction, but increased more rapidly above RI 1.3600.

Comparisons between runs was also facilitated by the normalization of results in which the content of a component in each fraction is expressed as a percentage of the total amount of the component recovered from the gradient. The efficiency of the separation was checked by calculating the recovery of each component as a percentage of the amount of it applied to the density gradient at the commencement of each run. Recoveries were close to 100%.

Evaluation of synaptic vesicle numbers

Density gradient fractions. Vesicles were counted in fractions by a modification of a previously described method[27]. They were fixed by treating a 1-ml sample of each fraction immediately after collection with 1 ml of cold (4 °C) 2% glutaraldehyde in 0.4 M sodium cacodylate buffer, pH 7.4 (SCB). After varying periods of storage, during which vesicles appeared to be stable, fractions were further processed for electron microscopy by adding 2 ml of SCB and 1 ml of 4% aqueous OsO_4. After 1 h, the mixture was centrifuged for 1 h at 100,000 g_{av} in a Beckman SW 50 rotor; the pellet so obtained was gently resuspended in 1 ml of SCB using a hand operated Dounce homogenizer with a loosely fitting pestle and the particulate material in the suspension collected by filtration on a 5-mm diameter Millipore filter of 0.45 μm pore size. The filter was washed with 1 ml SCB, suction stopped and 0.1 ml of 3% liquid agar poured over the filter to form a protective film. On cooling, the filter was detached, bisected and the two halves processed for electron microscopy by dehydration in ethanol and embedding in Epon 812. Blocks were trimmed so as to provide a 3 mm length of a vertical section through the vesicle pellet and a 1 μm section was cut and stained with Toluidine blue for a light microscopic determination of pellet thickness. This was done by taking the mean of 10 measurements along the length of the section with an ocular micrometer at a magnification factor of 400.

Thin (120 nm thick) sections were then cut from the same piece for electron microscopy. They were mounted without support on 150 mesh grids and counterstained with uranium and lead. A series of 5–10 micrographs were made of randomly selected areas along the 3 mm length of the pellet section at a magnification of 24,000 and prints were made from these with an additonal 3-fold enlargement. Profiles were counted and the number of vesicles per unit volume deduced by correcting for vesicle centres falling outside the section[1]. The multiplication factor is such that one vesicle per print corresponds to 10^8 vesicles·ml^{-1} of fraction. Samples containing much protein were difficult to embed and cut; for this reason no attempt was made to count vesicles in the cytoplasmic extract or to evaluate vesicle recoveries.

Tissues. The number of vesicles present within nerve terminals in intact tissue was counted in electron micrographs of sections cut vertically through stacks of electrocytes. The relative volume of nerve terminals was calculated from measurements of the fraction of the area of micrographs occupied by nerve terminal profiles.

RESULTS

Morphological and biochemical characterization of gradient fractions

All sealed vesicular profiles were counted independently of size or shape. The mean yield of isolated vesicles ($\times 10^{-13}$) per run was 1.58 ± 0.23 or 0.32 ± 0.09

per pg of tissue. Few or no vesicles were present in the early fractions; these were rich in soluble cytoplasmic proteins[38] (Fig. 1) and somewhat difficult to section. Vesicles were first detected at about fraction 18 (90 ml; RI, 1.350) and peaked (Fig. 1, peak I and Table I) at RI 1.3584 ± 0.0002 (4), consistently one or two fractions beyond the peak of acetylcholine at RI 1.3577 ± 0.0006. The profiles in these fractions were identical in morphology to those of the main population of synaptic vesicles in nerve terminals; the mean profile diameter (Fig. 1, insert) was close to those previously reported[15-18,27,28,41,42], using transmission electron microscopy and ultrathin sections[16,17,27,28,41,42] or negative staining[28], or a non-invasive biophysical method[15,16]. There were, in this region of the gradient, few profiles over 120 nm in diameter (Fig. 1, insert). Vesicles in small numbers were found all the way down the gradient and formed two smaller peaks (Fig. 1, II and III) at about RI 1.3670 and 1.3780 respectively. These denser fractions contained vesicular profiles up to 1400 nm in diameter as well as non-vesicular contamination. As previously noted[7], peak II coincided approximately with a peak of thiamine pyrophosphatase activity (Table I) and may thus have contained vesicles associated with, or derived from Golgi membranes, since thiamine pyrophosphatase is a recognized marker for such membranes. Peak III contained dense-cored vesicles in addition to those with electron-translucent cores; the mean profile diameter was 115 nm (Fig. 1, insert) and the peak coincided (Table I) with one of a polypeptide immunochemically resembling porcine vasoactive intestinal polypeptide (VIP)[2,3]. This peptide is known to be present, in many tissues, within cholinergic neurons and to be packaged in large dense-cored vesicles[3,5].

Synaptophysin (also known as p38), a recognized marker for synaptic vesicles[20,39] and probably for other storage particles as well[3], was also distributed right down the gradient; it formed two peaks, one coinciding with peak I of synaptic vesicles and the acetylcholine peak, the other approximately with peak III and the peak of

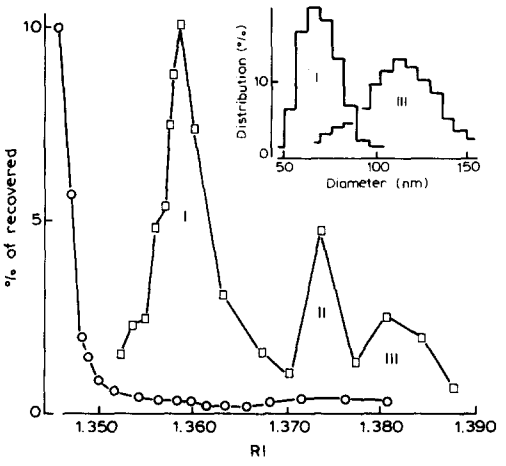

Fig. 1 Distribution in a zonal density gradient of (squares) vesicle numbers and (circles) protein. Insert: distribution of vesicle diameters in peak fractions of peaks I and III (from Agoston et al.[7]).

electromotor VIP (Table I). Although it was present in the region of vesicle peak II, it formed no definite peak in that region of the gradient.

The distribution of acetylcholine in the gradient

Endogenous acetylcholine. Acetylcholine formed, as expected, a single peak in the gradient with an RI of 1.3577 ± 0.0006 (4); the slightly lower value [1.3552 ± 0.0002 (16)] reported earlier[6] may have been due to a slightly lower operating temperature in the earlier runs. In some experiments (Fig. 2a,c) the peak was sharp and symmetrical; in others (Fig. 2b,d), a more or less pronounced shoulder could be seen on the dense side of the peak. When the number of acetylcholine molecules per vesicle (the molecular acetylcholine content) was calculated for each fraction by multiplying its molar acetylcholine content by Avogadro's number and dividing by the number of vesicles per litre, it became clear that the synaptic vesicles within peak I could indeed be grouped into at least 3 subpopulations or pools: a small number of light vesicles with a relatively low acetylcholine content (Fig. 2, V_0), a main population of vesicles

TABLE I

Biochemical characterization of vesicle fractions prepared by centrifugal separation in a sucrose–NaCl density gradient

Parameter	*Marker for*	*Mean RIs of peaks of vesicle distribution*		
		I	*II*	*III*
Vesicles[a]		1.3584 ± 0.0002(4)	1.3670 ± 0.0027(4)	1.3783 ± 0.0023(2)
Acetylcholine[a]	synaptic vesicles	1.3577 ± 0.0006(4)	–	–
TPPase[b]	Golgi membranes	–	1.3740	–
VIP-like immunoreactivity[b]	dense-cored vesicles	–	–	1.3783
Synaptophysin[b]	storage particles	1.3605	–	1.3783

[a] Values are means ± S.E.M. of 4 or ± range of 2 experiments in which measurements were extended to denser regions of the gradient.
[b] Results of Agoston et al.[7] converted to RI values.

highly charged with acetylcholine (Fig. 2, V_1) and a population of denser vesicles (Fig. 2, V_2), again with a lower acetylcholine content. The main V_1 subpopulation had a mean molecular acetylcholine content in satisfactory agreement with, though somewhat higher than that reported earlier[27] for the peak acetylcholine fractions. Since the fractions obtained in the earlier work had been worked up promptly for electron microscopy, this agreement suggests that some loss of vesicles may have occurred during storage and subsequent processing for electron microscopy but if so, this could not have been serious.

Vesicle counts did not, in general, show the smooth progression from fraction to fraction seen with acetylcholine and other parameters and there appeared to be a random fluctuation superimposed upon their distribu-

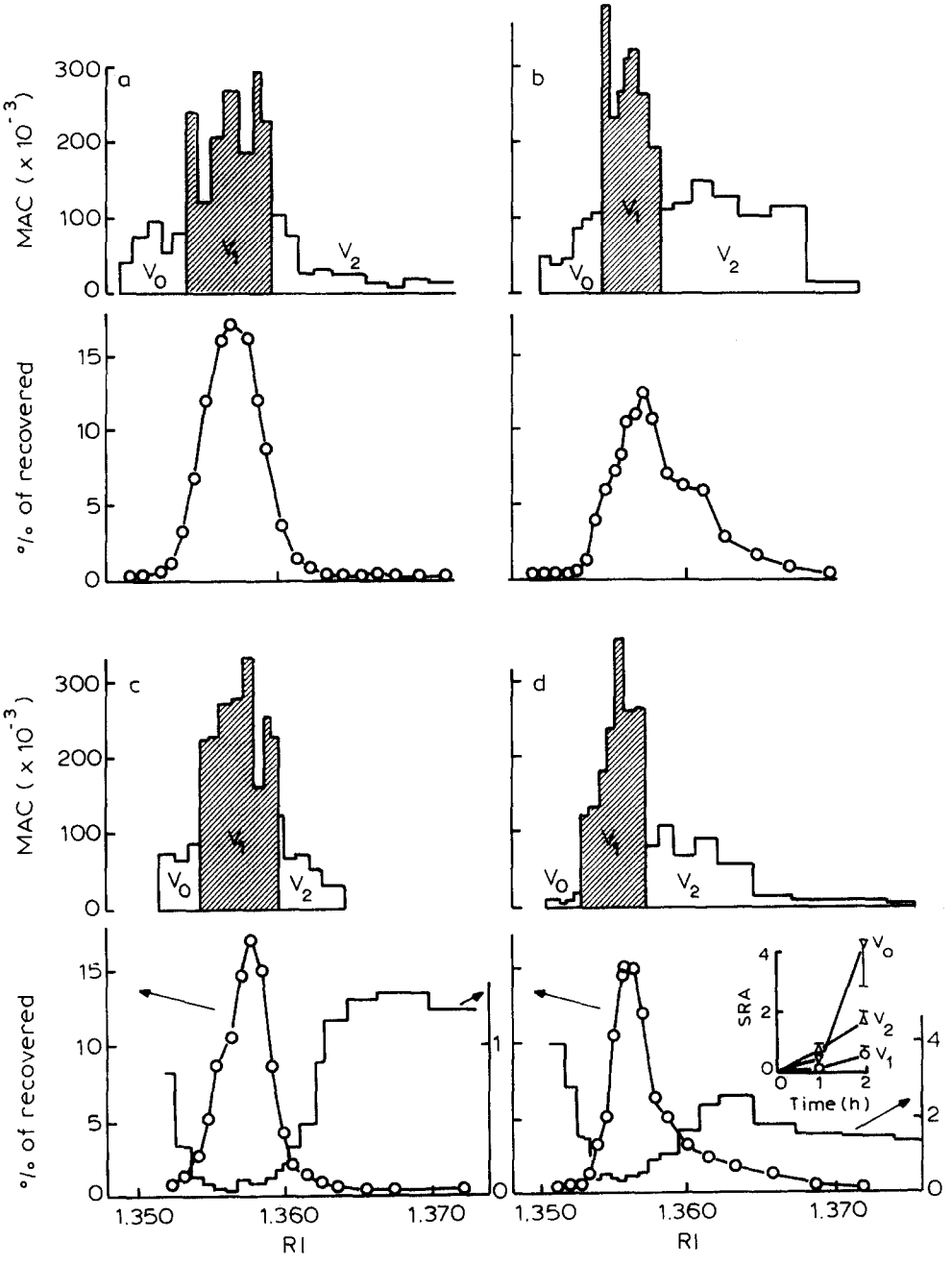

Fig. 2. Distribution in zonal density gradients of vesicular acetylcholine (circles) after extraction of vesicles from blocks (a,b) frozen immediately after dissection, or (c,d) after perfusion with [^{3}H]acetate for (c) 1, (d) 2 h. The upper histograms in a–d show the molecular acetylcholine content (MAC) of the vesicles in each fraction, the lower ones (c,d only) the specific radioactivity (SRA) of each fraction. Note that the scale of SRA in c is twice that in d. The insert in d shows the mean SRA of each vesicle subpopulation as a function of time. The results permit classification of the vesicles into 3 subfractions differing in density, acetylcholine content and ability to incorporate newly synthesized transmitter.

TABLE II

Acetylcholine content of subpopulations of synaptic vesicles isolated from unstimulated electric tissue

Block	*Perfusion time (h)*	*Percentage (a) and molecular content ($\times 10^{-3}$) (b) of acetylcholine in synaptic vesicle subpopulations*					
		V_0		V_1		V_2	
		(a)	*(b)*	*(a)*	*(b)*	*(a)*	*(b)*
a	0	5	70 ± 9(5)	88	223 ± 22(7)	7	36 ± 11(9)
c	1	5	76 ± 7(3)	86	245 ± 19(8)	9	73 ± 19(4)
b	0	7	73 ± 11(6)	67	285 ± 23(7)	26	127 ± 6(7)
d	2	1	13 ± 3(4)	79	229 ± 28(8)	20	81 ± 8(5)
All	–	4.5 ± 1.7	58 ± 15	80 ± 5	246 ± 14	16 ± 5	79 ± 19
Ohsawa et al.[27] (acetylcholine peak fractions)		–	–	–	260 ± 58(5)	–	–

tion in the gradient. The cause for this was not traced but was not due to counting errors, since independent counts made on any one set of electron micrographs usually agreed to within 10%. The fluctuations may have been caused by any or all of the following: random losses during processing, inhomogeneities within vesicle pellets and the smallness of the samples of the pellets taken for electron microscopy. Because of these fluctuations in the observed concentration of vesicles in the fractions, there was a fairly large variance in the mean molecular acetylcholine content within each population; however, differences between the V_1 and V_2 and between the V_1 and V_0 subpopulations were statistically significant ($p < 0.01$) (Table II).

Newly synthesized acetylcholine. In experiments in which radioactive acetate had been perfused, radioactive (i.e. newly synthesized) acetylcholine was taken up by all the synaptic vesicle fractions; however, when the specific radioactivity of the vesicular acetylcholine in each fraction was calculated, it became clear that the acetylcholine of both the V_0 and V_2 vesicles was considerable more radioactive than that of the fully charged V_1 vesicles (Fig. 2c,d). As expected, the amount of labelling of each fraction was greater after 2 h of perfusion than after 1 h (Fig. 2d, insert). Recent evidence suggests that vesicular acetylcholine uptake is regulated by the vesicles' internal content of acetylcholine and that uptake into replete vesicles is severely reduced[19,26,32].

DISCUSSION

Characterization of synaptic vesicles

The V_1 pool. The molecular acetylcholine content of the vesicles in the main portion of the acetylcholine peak is very close to that found in earlier work (Table II). Some very high values in individual fractions could be due to adventitious losses of vesicles during storage or preparation for electron microscopy. Stages at which losses or errors could have occurred include: breakdown during handling of vesicles rendered fragile by fixation, inhomogeneities in vesicle pellets inadequately revealed by the very small samples represented by the ultrathin sections viewed in the electron microscope, and inadequacies in the stereological routines which were a little different from those previously used[27]. It proved impossible to control all these factors, but, even assuming a percentage or fixed loss from each fraction, the statistical differences between V_1 and the two other pools would remain. The osmotic load of reserve vesicles has been estimated by a non-invasive biophysical technique[15] to be 0.21 aosmol per vesicle; this is consistent with the previously observed data[27] on the basis of an assumed activity coefficient of 0.65, a not unreasonable value for the concentrated solution of acetylcholine and ATP present in the vesicle core.

Recycling vesicles are present in unstimulated tissue. It seems clear, then, that two (V_1 and V_2) of the 3 subpopulations of synaptic vesicles here demonstrated to be present in resting blocks of electric organ tissue can be identified with the reserve (VP_1) and recycling (VP_2) vesicle pools previously shown to be present in stimulated tissue[6,18,41]. Vesicles isolated from resting tissue, though apparently homogeneous on the basis of the unimodal distribution of acetylcholine in the gradient and the relative symmetry of its peak, always show heterogeneity with respect to the incorporation of radiolabelled acetylcholine when labelled transmitter precursors are perfused[41,44]. This takes the form of a rising specific radioactivity on the descending, dense limb of the acetylcholine peak and has been held to show that recycling vesicles are also present in resting tissue. These may result from spontaneous quantized transmitter release (electromotor nerve terminals generate miniature postsynaptic potentials[11,12,30]), occasional discharges by

TABLE III

Proportion of subpopulations of synaptic vesicles in the total population isolated

Block	*Percentage of synaptic vesicles in*			*100 V_2/ (V_1 + V_2)*
	V_0	V_1	V_2	
a	11	64	25	28
c	12	65	23	26
b	16	46	38	45
d	12	37	51	58
Mean ± S.E.M.	13 ± 1	53 ± 7	34 ± 6	39 ± 7

the living fish, or adventitious discharges during dissection, the reality of which can be testified to by anyone who has carried out dissection with bare hands! Heterogeneity has also been demonstrated with respect to acetylcholine uptake in vitro[32].

Although V_2 vesicles could be readily distinguished from V_1 vesicles on the basis of their lower transmitter content and greater density, they represent a dynamic population with a gradient of densities and transmitter contents. Though the gradient failed to resolve subclasses within the V_2 fraction there is sufficient heterogeneity with respect to acetylcholine content to accommodate the phenomenon of subminiature postsynaptic potentials, if indeed these occur at the electromotor synapse (the evidence is equivocal; compare refs. 22 and 24).

One might expect that perfusion would give time for V_2 vesicles to become replete and join the V_1 pool as has been demonstrated for stimulated tissue[6,18,41]; this, however, is a slow process[40] and takes up to 16 h to be completed[6]. In the present series of experiments perfusion was carried out only for sufficient time to allow for labelling of the recycling pool.

That the size of this pool in resting tissue may be largely determined by the amount of adventitious stimulation the latter receives during the experiment seems to be borne out in the present series of experiments by the variations seen in the partition of synaptic vesicles between the V_1 and V_2 pools in identically handled blocks (Table III). The proportion of the V_1 + V_2 vesicles in the V_2 pool varied from 26 to 58%. The size of the V_2 pool was not so evident from the distribution of acetylcholine between the two pools (Table II), since only 7–26% of the transmitter is recovered in it and only at the upper end of this range is its presence revealed by a shoulder in the otherwise symmetrical acetylcholine peak.

Significance of the V_0 pool. A low-density vesicle fraction isolated from electromotor nerves but lacking acetylcholine was demonstrated[21,32] in pulse-labelling

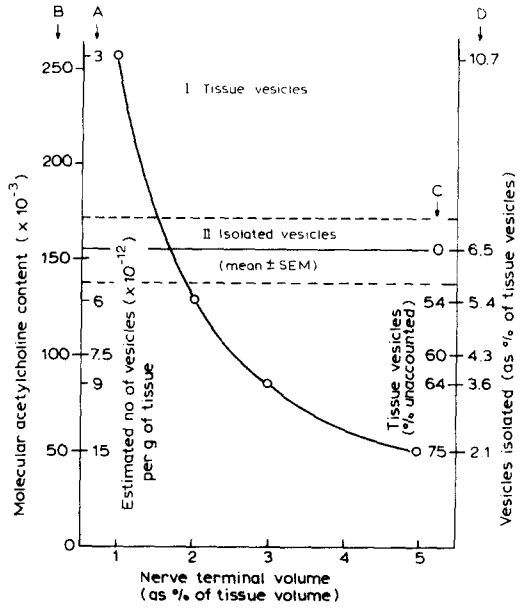

Fig. 3. Curve I shows (on ordinate scale A) the estimated no of vesicles per g of tissue and (scale B) the mean molecular acetylcholine content of tissue vesicles based on an estimate of 3 × 10^{14} vesicles·ml^{-1} of nerve terminal and a range of assumptions (abscissae) as to the vol-% of the tissue occupied by nerve terminals. Line II is the mean molecular acetylcholine content of isolated vesicles. It can be seen that if nerve terminals occupy 1.7 ± 0.2% of the tissue (a not unreasonable assumption) there are no tissue vesicles unaccounted for (scale C) in the sample isolated, which represents (scale D) 6.5% of the tissue vesicles. The calculations are based on a mean tissue acetylcholine content of 1.28 μmol (7.68 × 10^{18} molecules)·g^{-1} (or ml^{-1}) of tissue and a yield of isolated vesicles of 3.2 × 10^{11} per g of tissue.

experiments with ^{35}S to be also present in the electromotor nerve terminals where it took up acetylcholine and, in some hours, acquired the density of replete (VP_1) vesicles. Partial confirmation of these findings came from the isolation from electromotor axons of vesicles of similar density and very low acetylcholine and ATP content[7]. The V_0 vesicles in the present study had a similar density; they also (Fig. 1c,d) avidly took up newly synthesized acetylcholine in situ, and are thus probably to be identified with the VP_0 pool of axonal synaptic vesicles that have recently arrived in the nerve terminal. The contribution of these to the total synaptic vesicular pool is however quite low (Table III, 13 ± 1(4)%) and less than 5% of the vesicular acetylcholine is on average associated with them (Table II).

Vesicle diameters. In previous work[18,40-42] the mean diameter of vesicles of the pooled VP_2 fractions as deduced from stereological transformation of the distribution of vesicle profile diameters has been found to be about 25% less than that of similarly pooled VP_1 vesicles. This is consistent with a biophysical model of the vesicle[15] deduced from measurements of the density and water

content of vesicles of different degrees of osmotic loading, in which it is regarded as an osmometer with an aqueous core containing dissolved solutes (acetylcholine and ATP) and surrounded by a membrane selectively permeable to water. No repeat comparisons of vesicle diameters were made in the present work since (a) the density gradient was not iso-osmotic throughout its length and (b) in contrast to previous work vesicles were kept in fixative sometimes for a considerable time before processing (see Methods) and would be expected to have equilibrated with the suspension medium. Under these conditions[14] it was felt that any attempt to detect small differences in diameter between fractions would be unlikely to succeed.

Are isolated vesicles representative of the tissue vesicle population?

The isolation of an organelle in a high state of purity and homogeneity, like that of an enzyme or other protein, entails losses, and most isolation procedures are a compromise between yield and purity. Because of the high collagen content of the electric organ, its comminution requires vigorous methods, such as the freezing and crushing procedure originated by Whittaker et al.[38]. About 50% of the total tissue acetylcholine remains bound (presumably vesicular); of the 50% lost, about 40% has been shown, by a non-invasive technique[35] to be free, cytoplasmic acetylcholine. In the subsequent fractionation there are losses due to leakage of acetylcholine from the bound state which are revealed when recoveries fall below 100% and losses of bound acetylcholine along with discarded coarse tissue fragments in the P_{12} fraction. Just under 50% of the bound acetylcholine was originally recovered in the cytoplasmic (S_{12}) extract[38]. In later work in which, in order to reduce the amount of soluble cytoplasmic protein extracted, the ratio of the amount of crushed tissue extracted to the volume of extraction medium was increased and the temperature of extraction lowered, the proportion of bound acetylcholine was lower (27%)[41]. Recoveries from the zonal gradient are usually satisfactory (98 ± 3% in the present series). Thus, although the percentage of total tissue acetylcholine or of the estimated amount of tissue vesicular acetylcholine recovered from the gradient is quite low, losses during fractionation can be precisely accounted for and there is no reason to suppose that what is isolated is not a fully representative sample of the original vesicle population. This proposition may in principle be tested in the following way.

The estimate of the tissue content of vesicular acetylcholine made by a non-invasive technique[35] when divided by the estimated number of vesicles in unit weight of tissue gives us the amount of acetylcholine per vesicle. This may then be compared to the average acetylcholine content per vesicle of the isolated vesicles.

Unfortunately it is not a trivial matter to determine the number of vesicles per unit weight of tissue, since this has to be done by stereological methods from conventional electron micrographs and it is difficult to sample the tissue as a whole or to control for the amount of shrinkage which occurs when the tissue is prepared for electron microscopy. Using the procedure described in Materials and Methods I obtained the value of 303 ± 30 (11) vesicles·μm^{-3} for nerve terminals. This is in the range obtained by Fox et al.[13] (300–325). In sections through stacks of electrocytes, nerve terminals occupy about 5% of the volume of the fixed, embedded and sectioned tissue but this does not take into account the morphology of the edges of the electrocytes, where innervation is reduced, the proportion of the volume of the tissue taken up by the nerve trunks, the blood vessels, and the extracellular space between the electrocyte stacks, nor the effect of tissue shrinkage during dehydration and embedding. Thus a value of half or less of this might be more reasonable. Fig. 3 gives (curve I, scale A) the number of vesicles·ml^{-1} of tissue for a range of assumptions (abscissae) concerning the proportion of the whole tissue occupied by nerve terminals, based on the estimate of 3×10^{14} vesicles·ml^{-1} of nerve terminal.

The acetylcholine content of the tissue samples from which the electron micrographs were made is not known and published values vary over quite a wide range. However, Weiler et al.[35], using gas chromatography, obtained a mean value of 1.64 μmol·g^{-1} of which 22% was estimated to be cytoplasmic by a non-invasive technique. This gives for vesicular acetylcholine, 1.28 μmol·g^{-1} of tissue or 7.68×10^{17} molecules·g^{-1}. The estimated mean molecular acetylcholine content per vesicle thus ranges (Fig. 3, scale B) from 51,000 to 256,000 depending on the assumptions made. This may be compared to 155,000 ± 17,000(4) as the mean value for the isolated synaptic vesicles (Fig. 3, line II). Clearly this leaves no room for large numbers of tissue vesicles that remain unaccounted for by the sample isolated as has been maintained by Fox et al.[14], even though this may represent only 2–10% of the tissue vesicle population (scale D), again depending on the assumptions made. In scale C, the proportion of empty vesicles unaccounted for in the sample isolated is calculated. It ranges from 75% of the tissue vesicle population if 5% of the tissue is nerve terminals to zero if it is 1.7%. If it is only 1%, we should have to assume that all the vesicles in the tissue are fully charged with transmitter to the level observed in the V_1 fraction and that a considerable proportion of them had lost part or all of their acetylcholine during isolation, which is not borne out by the recoveries during fraction-

ation. It thus seems implausible that there is a class of empty vesicles in the tissue which is selectively destroyed by freezing, extraction etc., or selectively left behind in the discarded fractions and so not represented in the isolated vesicles as proposed by Fox et al.[14].

What these calculations also serve to show is the difficulty of making quantitative estimates based on the small samples provided by the highly invasive technique of conventional electron microscopy of whole tissue.

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