Biochemical Pharmacology, Vol. 27, pp. 3493–3498.

COMMENTARY

ARE VESICLES NECESSARY FOR RELEASE OF ACETYLCHOLINE AT CHOLINERGIC SYNAPSES?

LADISLAV TAUC

Laboratoire de Neurobiologie Cellulaire, Centre National de la Recherche Scientifique 91190 Gif sur Yvette, France

The view that in a process analogous to that used by gland cells, synaptic vesicles are involved in the transport of the transmitter from the axoplasm into the synaptic cleft across the lipid bilayer membrane of the terminal presynaptic membrane, was initially based on the necessity to find a way in which the transmitter can be released in molecular packets. In the original formulation of the 'vesicular hypothesis', Del Castillo & Katz [1] assumed that the recently discovered synaptic vesicles contain the transmitter substance and that when they, in their random movement, touch the presynaptic membrane at critical contact zones, they discharge their content by exocytosis; the probability of contact increases with nerve stimulation. This was not only a satisfactory explanation of the occurrence of both miniature end-plate potentials (mepp) and of the quantal character of the evoked end-plate potential (epp) [2] but it also explained how the quanta are formed and stored in the nerve terminal for ready release.

The demonstration that in cholinergic neurons synaptic vesicles effectively contained ACh [3, 4] considerably asserted the vesicular hypothesis; since then no other concept of transmitter release has been seriously developed as it seemed absurd to consider any other than vesicular transport.

In the last few years, however, observations were accumulated which seriously question the validity of the vesicular hypothesis: ACh was found not only confined in vesicles but also 'free' in the cytoplasm at a concentration which would permit its release through a specific channel following its concentration or electrochemical gradient; such release would also be quantal; the cytoplasmic ACh, and not the vesicular one is renewed during stimulation; enzymatic destruction of the cytoplasmic ACh blocks synaptic transmission; the replacement of ACh by a 'false' transmitter does not lead at intermediate stages to a bimodal distribution of 'false' and 'true' mepp; membrane modifications which were attributed to exocytosis of synaptic vesicles seem to appear too late to be responsible for ACh release. These and some other difficulties which face the vesicular theory would justify the elaboration and experimental testing of another model for the transmitter release. It appears however that the first step necessary to initiate such a research is to remove from the vesicular hypothesis its aura of absolutism and place it at the same rank as other concepts present and to come. The aim of this article is to present a short critical survey of the vesicular hypothesis. It is limited to cholinergic synapses, the only system where enough experimental data are available to seriously discuss this matter.

ACh IS PRESENT IN SYNAPTIC VESICLES AND IN THE TERMINAL AXOPLASM

The vesicular and extravesicular concentration of acetylcholine (ACh) was extensively studied in the electric organ of *Torpedo*, since this organ has a homogenous and very dense cholinergic input. It was possible, using density gradient fractionating methods, to isolate synaptic vesicles [5–8] and pinched off presynaptic terminals (synaptosomes) were successfully prepared [9–11]. By correlating the volume of nerve terminals of the electric organ [6, 12] or of the synaptosomes [13, 14], with the total ACh content, it was possible to estimate the mean presynaptic concentration of ACh to be about 20–27 mM in both cases. Two nearly equal compartments of ACh were defined biochemically [15], designated as 'free' which in a homogenate is exposed to hydrolysing action of acetylcholinesterase (AChE) and destroyed, the other one 'bound' and protected from the underestimation of the number of vesicles found to be equivalent to vesicular ACh. It was estimated that the concentration of ACh in the vesicles corresponds to about 70–80 mM, and that in the 'free', most probably axoplasmic compartment, ACh is present at a concentration of about 10 mM.

Higher values (500–600 mM) for ACh vesicular concentration were given by Whittaker *et al.* [7] and Wagner *et al.* [16]; possibly these high figures result from the underestimation of the number of vesicles taken into account. Anyway these values are the mean, supposing a homogenous vesicular population.

Another evidence of the presence of cytoplasmic ACh was given by Birks [17] who found that in the sympathetic ganglion stimulated for 20 min, the amount of synaptic vesicles decreased to a half, whereas, because of active synthesis, the total amount of ACh in the ganglion did not change. Either each vesicle now contained twice as much ACh as at rest or ACh was now present in the cytoplasm at a concentration of 50 mM or more. This second alternative seems more plausible, as the vesicular concentration of ACh at rest was already estimated to be at least 750 mM.

At the neuromuscular junction, ACh outflow at rest is about 200 times higher than that which would be expected from the spontaneous discharge resulting in mepp [18, 19]. In fact, this can be explained by a steady leakage of molecular ACh from the terminal, as demonstrated by Katz & Miledi [20]. Local injection of *d*-tubocurarine on the resting frog anti-esterase treated nerve end-plate induced a small local hyperpolarization in the postsynaptic muscle fibre resulting most probably from the blockage of

postsynaptic ACh receptors, continuously activated by a presynaptic non quantal leakage of cytoplasmic ACh. It was assumed that the cytoplasmic concentration of ACh was approximately 27 mM. Similar results were obtained by Vyskočil & Illes [21] at mouse neuromuscular junction.

After all, it is not surprising that ACh is present free in the cytoplasm of the terminal. ACh synthetizing enzyme, choline acetyltransferase, is a cytoplasmic, not vesicular, enzyme. It is important to know that ACh concentration in the cytoplasm reaches values which make possible to consider the reality of a release mechanism in which the transmitter would not necessarily need to go through vesicular confinement.

VESICLES AND ACh AT STIMULATED SYNAPSES

Although there seems to be a general agreement that vesicle number is reduced following intense stimulation of synapses [12, 17, 22–27], there is not a simple correlation between the number of vesicles and the amount of ACh released [12, 17]. The situation is still more complicated when the vesicular content of ACh is considered. After having applied a strong tetanic stimulation to the *Torpedo* electric organ, Zimmerman & Whittaker [25] have analysed the recovery from fatigue; they found that the number of vesicles recovered in 4 hr, the global electrical response in 5 hr but the vesicular ACh concentration in 3 days. The total ACh was unfortunately not measured. It is clear that such a result cannot be explained in classical terms of vesicular hypothesis. Zimmerman & Whittaker proposed a model in which only a small part of vesicles effectively participates to release. In later work [28–30], Whittaker's group claims to have separated, by density gradient centrifugation, the vesicular population into two fractions: one monodisperse, less dense, pure vesicular fraction VP_1, and a second denser fraction VP_2, in which the vesicles are associated with membrane fragments, supposedly mainly derived from the cleft region of the presynaptic membrane. When an ACh radioactive precursor was added to the organ prior to the stimulation [28], it was this VP_2 fraction which showed the highest specific activity indicating large incorporation of labelled ACh. Whittaker's group thus attributed to the vesicles in the VP_2 fraction the role of the active vesicular subpopulation supposedly involved in transmitter release by exocytosis.

But already several years ago, Israël's group [12, 31, 32] published the work on the evolution of the two ACh compartments when the *Torpedo* electric organ underwent repetitive stimulation in the presence of labeled precursors. Six minutes stimulation at 10/sec depressed the electrical response to nearly zero. Surprisingly enough (at least in the view of the vesicular theory), at the end of the stimulation period, the number of vesicles remained constant, the amount of vesicular ACh was unchanged and the absence of any change of specific radioactivity indicated that no new ACh was incorporated into the vesicles. On the contrary, cytoplasmic ACh decreased to half of its initial value and its high specific radioactivity pointed to an intense renewal of ACh in this compartment.

These results showed clearly that the newly synthetized ACh was released preferentially (in agreement with previous observations on sympathetic ganglia [33]) and that the nerve stimulation depleted and turned over cytoplasmic ACh which was not transfered to the vesicles.

In a recent controversial discussion between the two groups, Dunant & Israël [34] deny the existence of specific active vesicles and consider that ACh of high specific radioactivity detected by Suszkiw *et al.* [28] in the fraction VP_2 was there because of the 'survival' of cytoplasmic ACh (not determined by Suszkiw *et al.* [28]) which was more or less specifically associated to membranes heavier than the synaptic vesicles. Comparison between the experimental values given by the two groups [15, 28] show indeed that during stimulation vesicular ACh remained stable, whereas cytoplasmic ACh and ACh in the VP_2 fraction were modified identically.

It is clear from these results that a vast majority of synaptic vesicles do not participate in transmitter release; remembering the formulation of the vesicular hypothesis, one can question whether it is appropriate that the 'new look' vesicular hypothesis of Whittaker's group bears that label.

EXOCYTOSIS OF SYNAPTIC VESICLES

Morphological changes at stimulated synapses would be expected if exocytosis model was applicable. Modifications of the nerve terminal membrane were actually observed at the heavily stimulated frog neuromuscular junction. Invaginations and membrane foldings were described [23, 35–37]. Moreover, horseradish peroxidase [36] and acetylcholinesterase [38] were found in a population of synaptic vesicles following a prolonged stimulation. These changes were interpreted as indicating the fusion and incorporation of synaptic vesicles with the terminal membrane and the reformation of these vesicles through retrieval of the cytoplasmic membrane forming coated vesicles which coalesce into cisternae and divide later to form new synaptic vesicles ('the recycling hypothesis'). Without entering into the classical controversy regarding the interpretation of anatomical data, it is obvious that the eventual reality of exocytosis of synaptic vesicles does not provide the evidence that the given vesicles contained ACh. The fact that vesicles can be charged by an extracellular marker indicates that they can contain other substances than ACh. Stimulated terminals accumulate undesirable ions and catabolites; vesicles could be good organelles to get rid of them.

That the content of an exocytosing vesicle might be different from the content determined by biochemical studies of isolated synaptic vesicles, appears from the measurement of ATP outflow from stimulated synapses. It was established that synaptic vesicles contain not only ACh but also ATP, ACh being three to five times more concentrated than ATP [16, 39, 40]; yet no release of ATP was observed in stimulated sympathetic ganglia [41], nor was the ACh release accompanied by the release of adenoside nucleotides in the chemically stimulated *Torpedo* synaptosomes [80]. This is contrary to hormone secretion, where it was shown that ATP contained

in the secretory vesicles is correleased with the hormone [42, 43]. Thus, admitting the exocytosis of synaptic vesicles exists, the content of these vesicles may be different from that of the vesicular mass, either because the vesicular population is heterogenous (for instance vesicles containing ACh do not contain ATP) or the content of a vesicle has changed prior to its fusion. The proposal that they really contain ACh is thus mostly conjectural.

IS EXOCYTOSIS OF SYNAPTIC VESICLES CONTEMPORARY TO ACh RELEASE?

If ACh is extruded by the fusion of vesicles with the presynaptic membrane, one would expect that the corresponding invaginations would be contemporary to transmitter release. To ascertain this, Heuser *at al.* [44, 81] have developed a sophisticated technique which permitted to freeze, in a fraction of a msec, the frog neuromuscular junction a short time after it was stimulated. 4-Aminopyridine was added to the preparation to considerably increase the postsynaptic response. Effectively, figures of exocytosis were seen in freeze fraction views after a single stimulation, but not earlier than 5 msec after the stimulus was delivered. However, the experimental conditions were such that most of the transmitter would be discharged within 3 msec, taking into account the conduction of spike and the synaptic delay, but at 3 msec, no pits were apparent [44, 81]. Thus, on the basis of data so far published by Heuser *et al.*, it appears that the exocytotic figures are posterior to ACh release for which it is difficult to see how they could be responsible.

There are many theoretical difficulties to understand vesicle fusion with cytoplasmic membranes [45, 46]. One such aspect was discussed by Parsegian [47] and concerns the repulsive forces between phospholipid membranes. The vesicle, in order to approach the membrane and to fuse, has not only to overcome electrostatic repulsive forces (which would be eventually bridged by Ca^{2+}), but below about 30 Å separation, encounters the force of hydration, representing the work of transfer of water-soluble polar groups that stabilize membrane surfaces. With further approach, this repulsive force grows to such a value that to allow approach of membranes and subsequent membrane fusion without internal loss of vesicle content, it appears necessary to remove some hydrophylic groups from both vesicles and the cytoplasmic membrane by an enzymatic, presumably phospholipase, mediated process. Alternatively the stabilizing polar group could be pulled away from the place of membrane confrontation by contractile proteins, like neurostenin [48].

In both cases, the time necessary to perform such an operation would be of the order of a millisecond. Or does the synapse possess so much time to release the transmitter?

Llinas and his co-workers [49] measured with an improved voltage clamp technique, the time which elapses between the calcium entry in a stimulated terminal of the squid giant synapse and the start of the postsynaptic potential. This time was 200 μsec, during which period the calcium has to reach the strategic sites inside the nerve terminal and activate the release mechanisms, the transmitter has to cross the 200 Å of the synaptic cleft, and bind to postsynaptic receptors, and finally the ions have to start crossing the opened postsynaptic channels. Only a fraction of these 200 μsec is devoted to the activation of the release mechanism. This time left is several times shorter than that necessary to perform any enzymatic or contractile protein intervention known to permit vesicular fusion. Moreover, Llinas [49] points out that squid giant synapse is not the fastest synapse known; for instance, the rat neuromuscular junction is much more rapid [50]. The time requirements for the vesicular fusion possibly explain why Heuser & Reese were unable to observe figures of exocytosis in the first milliseconds. The actual transmitter release process is much faster than our present knowledge of fusion mechanisms would permit.

No such problems exist for the extrusion of secretory material from glandular structures, as there the system functions in a much slower mode and the fusion of vesicles has all time needed for known processes to operate.

BLOCKING OF THE TRANSMISSION WHEN CYTOPLASMIC ACh IS HYDROLYSED BY AChE

Landmesser and Pilar [51] studied the onset and development of transmission in the chick ciliary ganglion. They found that when the ganglion cells were fully transmitting at stage 33.5 the synapses did not contain synaptic vesicles. How could ACh be released from vesicles which did not exist?

If transmission is effective without synaptic vesicles, one would expect that if cytoplasmic ACh is absent, transmission should be impossible. This type of approach for the identification of releasable ACh was tried on a cholinergic neuronal couple in the buccal ganglion of *Aplysia* by artificially introducing AChE into the presynaptic neurone and controlling the transmissions across the synapse by measuring the psp[52–54]. About 2 hr after intracellular injection of AChE the psp started to decrease and eventually disappeared. Histochemical control revealed that the delay of the AChE action was due to the transport time of the enzyme down to the nerve terminals distant about 300–500 μm from the cell body. After the blocking of the synapse, electron micrographs have shown that the presynaptic vesicles were present in apparently unchanged numbers and clear of AChE. This was expected as it is known that AChE even at high concentration does not penetrate in the synaptic vesicles and does not destroy vesicular ACh [6].

To decide whether the depression of the evoked psp is due to a depletion of vesicular ACh or to a decrease of cytoplasmic ACh, the synapse was repetitively stimulated for 10 min when, after the injection of AChE, the psp was depressed to about a half of its original amplitude. Two effects of such stimulation could be expected: (1) if the vesicular hypothesis is true, the stimulation should accelerate the rate of depression of the psp, this because the number of ACh charged vesicles is limited, exchange between vesicular and cytoplasmic ACh is very slow, and because of the impossibility of new vesicles to be recharged by cytoplasmic ACh, which at that time

was already hydrolysed by AChE. (2) If the vesicular hypothesis is wrong, the stimulation should not modify the rate of depression of the psp, the concentration of ACh in the cytoplasm resulting only from the equilibrium between ACh synthesis and hydrolysis by AChE, so that the rate of the decrease of the psp is governed by the increasing levels of AChE coming down into the terminal.

The experimental result was in agreement with the second hypothesis: the rate of decrease of the psp was unaffected by the stimulation. To ascertain that the blocking of the transmission was due to AChE on cytoplasmic ACh, a series of control experiments was performed. The possibility of the action of the injected enzyme on spike conduction was discarded by studying the ACh release with direct depolarization in the presence of tetrodotoxin; the specificity was established by the absence of action of injected AChE previously inactivated by an irreversible organophosphorus blocker, phospholine. Injection of radioactive ACh precursor into the interneurone led to an intracellular synthesis of labeled ACh, which could be chemically separated into free (probably cytoplasmic) and bound (probably vesicular) pools. Injection of AChE and the subsequent blocking of the ACh release induced a drastic diminution of the 'free' pool leaving the 'bound' pool unchanged. This can be interpreted as indicating that when the synapse was blocked by AChE, cytoplasmic ACh was destroyed but ACh was still present in the vesicles.

These results are in accordance with the concept that the vesicular ACh cannot be released directly into the synaptic cleft by exocytosis. Prior to its liberation, ACh has to be present in the axoplasm in a form in which it can be exposed to the hydrolysing action of AChE.

USE OF FALSE TRANSMITTERS

Some choline analogues can be taken up into the nerve terminal by a high affinity system, acetylated and eventually released on stimulation as false transmitter [55–57]. One of them, the acetyl-monoethylcholine (AMECh), when released, can be distinguished from ACh by the fact that the miniature end-plate currents in rat muscle due to AMECh have a shorter time constant of decay than that due to ACh. This is because postsynaptic channels opened by AMECh have a briefer lifetime than these opened by ACh [58]. When MECh was added to the rat phrenic nerve diaphragm and the preparation stimulated, endogenous ACh was replaced progressively by AMECh. In the terms of vesicular hypothesis, one would expect that at intermediate state of exchange new vesicles would be preferentially filled with AMECh and released together with older ones containing ACh. This should have given rise to bimodal distribution of miniature end-plate currents (mepc), the shorter due to AMECh, the longer due to ACh. Such was not the case [59]; on the contrary, there was a progressive shortening in the mepc indicating a continuous mixing of the 'true' and 'false' transmitter. If the mepc were produced by the extrusion of the content of a vesicle, it would mean that there exists a rapid exchange between the cytoplasm and vesicular compartments. This was the conclusion of the authors [59]. However as reported above, such rapid exchange between cytoplasmic and vesicular ACh was not observed in any biochemical study (cf. [32, 34]). Consequently, quanta of mixed 'false' and 'true' transmitters must have come from the cytoplasmic compartment.

One important observation pointing to an additional complexity to the problem of transmitter release, is that the incorporation of AMECh into the quanta released by nerve stimulation appeared to take place more rapidly than its incorporation into spontaneously released quanta. This points to a possibility that different pools of transmitter may be responsible for spontaneous and evoked release. In regenerating frog muscle fiber, Dennis & Miledi [60] have found that the mean amplitude of spontaneous mepp was smaller than that of quanta contributing to evoked endplate potentials. McLachlan [61] stimulated the hemicholinium treated guinea pig superior cervical ganglion (hemicholinium renders the synthesis of ACh impossible) and found that the evoked postsynaptic potential amplitude decreased much more rapidly than that of the simultaneously recorded miniature postsynaptic potentials. After prolonged stimulation, she could observe evoked psp's which were smaller than the spontaneously appearing mpsp's. A comparable situation was found by Bennett *et al.* [62] in the Mauthner fibre-giant synapse of the hatchetfish, where stimulations at high frequency depressed the evoked postsynaptic potential below the amplitude of the miniature psp.

This duality in evoked and spontaneous responses has not so far received satisfactory response: it may result from plurality of ACh compartments, from different local dynamical characteristics of the release system, from different localizations of the evoked and local responses, or in an extreme case from different release mechanisms.

CONCLUSION

Heuser [82] states that "the evidence in favour of synaptic vesicle exocytosis is overwhelming". This is perhaps true, but it is now doubtful that this exocytosis is responsible for the bulk of transmitter release. Moreover, it is impossible to maintain the original concept supposing a more or less random movement of a homogenous synaptic vesicle population. The new proposal that only a specialized small vesicular fraction participates effectively to the transmitter release, invalidates in fact many arguments thought to be in favour of the vesicular hypothesis.

If most of the vesicles are not the agents of ACh extrusion, what can be their role? As already mentioned, the vesicles might serve to pick up from the cytosol the unwanted subproducts of synaptic transmission. The most annoying type of waste is calcium, the cytoplasmic concentration of which must be kept low (10^{-7}, 10^{-8} M).

In stimulated synapses, calcium was shown to accumulate inside the nerve terminal close to the presynaptic membrane [63, 64] and calcium binding of synaptic vesicles was demonstrated in several instances [24, 65–67]. It is tempting to attribute to

the vesicles the role of sequestering and exocytosing calcium [34].

Analysing Llinas results on the squid giant synapse [49], Parsegian [47] estimates that about 1.7×10^9 Ca^{2+} ions flow into the presynaptic terminal during an action potential, and releases 5×10^3 quanta. Supposing that a quantum is formed of 10^4 molecules of the transmitter, it appears that about 30 times more calcium ions penetrate into the terminal than transmitter molecules leave the cell.

We can imagine that part of this calcium enters the vesicles and is evacuated by exocytosis. The vesicles close to the plasma membrane would be first affected. Yet the high affinity calcium binding axoplasmic system was found to be neither mitochondrial nor vesicular [68]. Thus possibly this mode of extrusion of calcium by synaptic vesicles is used only during a heavy entry of calcium (for instance prolonged stimulation, acidic formaldehyde fixation, stimulation in presence of 4-aminopyridine) where the elevation of Ca^{2+} concentration cannot be handled by normal pumping of exchange mechanisms and high affinity Ca^{2+} buffering systems are saturated. This would explain why in normally stimulated synapses the exocytosis pits are practically never found on electron micrographs [44].

Following this concept, the vesicles would serve as reserve of ACh which in more or less extreme cases they would exchange against calcium and extrude this latter by exocytosis. This function is not less noble than that leading to the release of the transmitter.

How otherwise may the transmitter be released than by the exocytosis of synaptic vesicles? A gate hypothesis was suggested by Birks [17] and proposed by Marchbanks [69–71]. It has to overcome three major difficulties. First, the release of a quantum is not electrogenic, as would be expected if cytoplasmic ACh diffused through a channel following its electrochemical gradient [72]. Second, the amplitude of the mepp remains constant whichever polarization is imposed on the terminal [73]; the quantity of ACh released as a cation would indeed be expected to change with the presynaptic membrane potential. Third, fast oscillations of cytoplasmic ACh content during nerve activity observed with a period of about 5 sec by Dunant *et al.* [74] are not accompanied by a change in the size of miniature end plate potentials.

As a consequence ACh release cannot result from a single opening of an ACh "specific" channel (see also [34, 75]). In a hypothetical portrait the structure involved in the release should be apposed to the inner face or be part of the presynaptic membrane; it should be a mechanism which binds ACh to saturation to explain the relative constancy of the quantal size; it must be charged from the cytoplasmic ACh pool, but sufficiently separated from this pool to release ACh in a non or only slightly electrogenic manner. Calcium remains naturally the trigger of the release, performed perhaps by some carrier mechanism or a restricted gate.

Dunant & Israël [34] proposed the term 'operator' to described such a release mechanism. Because functioning of this mechanism combines some properties usually attributed to vesicles (such as confinement of ACh) and others to a gate (membrane mechanism), I propose a more eloquent and a more coloured term 'vesigate'.

Speculation about the structural correlate of the vesigate might designate a membrane formation attached to the presynaptic membrane. This might be for instance part or the whole of the vesicular presynaptic grid, a geometrical combination of dense projections and depressions or 'holes' forming a hexagonal sieve, supposed earlier to represent a guiding device for synaptic vesicles to reach performed loci at the synaptic sites [76] and to help in dispensing the transmitter towards the receptive elements of the postsynaptic surface ('Synaptopores', [77, 78]).

It is fairly possible that Whittaker and Zimmermann [79] are right when they claim to have isolated in the VP_2 fraction (see above) the membrane 'channel'. However, these 'channels' are not necessarily vesicles with some special properties as they think, but the 'contaminating' membrane fragments which seem to be mainly derived from the cleft region of the presynaptic membrane. If these membrane fragments included saturated vesigates, it is expected that ACh in this VP_2 fraction showed the same specific radioactivity as the cytoplasmic pool, although no occluded cytoplasm was present. Alternatively the heavier vesicles might effectively be part of the membrane release mechanism from which they were separated by the fractionating method used.

Many questions remain without answer, especially why in some cases one sees a divorce between evoked and spontaneous release. Perhaps more than one mechanism of release coexists in the same synapse and it cannot be excluded that some of the exocytosing vesicles still contain acetylcholine.

REFERENCES

1. J. Del Castillo and B. Katz, in *Microphysiologie Comparée des Eléments Excitables.*, Coll. Internat. C.N.R.S. Paris No. 67, pp 245–258 (1957).
2. B. Katz, *The Release of Neural Transmitter Substances*, Liverpool University Press, Liverpool (1969).
3. V. P. Whittaker, I. A. Michaelson and R. J. A. Kirkland. *Biochem. J.* **90**, 293, (1964).
4. E. D. P. De Robertis, A. Pellegrino de Iraldi, G. Rodriguez De Lorez-Arnaiz and L. Salganicof. *J. Neurochem.* **9**, 23 (1962).
5. M. Israël, J. Gautron and B. Lesbats. *C. r. Acad. Sci.* **266**, 273 (1968).
6. M. Israel, J. Gautron and B. Lesbats. *J. Neurochem.* **17**, 1441 (1970).
7. V. P. Whittaker, W. B. Essman and G. H. C. Dowe. *Biochem. J.* **128**, 833 (1972).
8. S. S. Carlson, J. A. Wagner and R. B. Kelly. *Biochemistry* **17**, 1188 (1978).
9. M. Israel, R. Manaranche, P. Mastour-Frachon and N. Morel, *Biochem. J.* **160**, 113 (1976).
10. M. J. Dowdall and H. Zimmerman, *Exp. Brain Res.* **24**, 8 (1976).
11. D. M. Michaelson and M. Sokolovsky, *Biochem. biophys. Res. Commun.* **73**, 25 (1976).
12. Y. Dunant, J. Gautron, M. Israël, B. Lesbats and R. Manaranche, *J. Neurochem.* **23**, 635 (1974).
13. N. Morel, M. Israel, R. Manaranche and P. Mastour-Franchon, *J. cell Biol.* **75**, 43 (1977).
14. N. Morel, M. Israël and R. Manaranche, *J. Neurochem.* **30**, 1553 (1978).

15. Y. Dunant, J. Gautron, M. Israël, B. Lesbats and R. Manaranche, *J. Neurochem.* **19**, 1987 (1972).
16. J. A. Wagner, S. S. Carlson and R. B. Kelly, *Biochemistry* **17**, 1199 (1978).
17. R. I. Birks, *J. Neurocytol.* **3**, 133 (1974).
18. J. F. Mitchell and A. Silver, *J. Physiol., Lond.* **165**, 177 (1963).
19. P. Fletcher and T. Forrester, *J. Physiol. Lond.* **251**, 131 (1975).
20. B. Katz and R. Miledi, *Proc. r. Soc. Lond. B.* **196**, 59 (1977).
21. F. Vyskočil and P. Illes, *Pflügers Arch. Physic.* **370**, 295 (1977).
22. B. Ceccarelli, W. P. Hurlbut and A. Mauro, *J. Cell Biol.* **54**, 30 (1972).
23. J. E. Heuser and T. S. Reese, in *Synaptic Transmission and Neuronal Interaction* (Ed. M. V. L. Bennett), pp. 59–77. Raven Press, New York (1974).
24. H. Zimmermann and V. P. Whittaker, *J. Neurochem.* **22**, 435 (1974).
25. H. Zimmerman and V. P. Whittaker, *J. Neurochem.* **22**, 1109 (1974).
26. A. F. Boyne, T. P. Bohan and T. H. Williams, *J. Cell Biol.* **67**, 814 (1975).
27. J. J. Pysh and R. G. Wiley, *J. Cell Biol.* **60**, 365 (1974).
28. J. B. Suszkiw, H. Zimmermann and V. P. Whittaker, *J. Neurochem.* **30**, 1269 (1978).
29. H. Zimmerman and V. P. Whittaker, *Nature, Lond.* **267**, 633 (1977).
30. H. Zimmermann and C. R. Denston, *Neuroscience* **2**, 715 (1977).
31. M. Israël, B. Lesbats and R. Manaranche, *C.r. Acad. Sci.* **275**, 2957 (1972).
32. M. Israël and Y. Dunant, in *Metabolic Compartmentation and Neurotransmission*, (Eds. S. Berl, D. D. Clarke and D. Schneider), pp. 621–639. Plenum, New York (1975).
33. B. Collier, *J. Physiol. Lond.* **205**, 341 (1969).
34. Y. Dunant and M. Israël, *Trends Neurosci.* **2**, 130, (1979).
35. R. Couteaux and M. Pecot-Dechavassine, *C.r. Acad. Sci. Paris* **271**, 2346 (1970).
36. J. E. Heuser and T. S. Reese, *J. Cell Biol.* **57**, 315 (1973).
37. B. Ceccarelli, W. P. Hurlbut and A. Mauro, *J. Cell Biol.* **57**, 499 (1973).
38. A. Politoff, A. L. Blitz and S. Rose, *Nature Lond.* **256**, 324 (1975).
39. M. J. Dowdall, A. F. Boyne and V. P. Whittaker, *Biochem. J.* **140**, 1 (1974).
40. H. Breer, S. J. Morris and V. P. Whittaker, *Eur. J. Biochem.* **80**, 313 (1977).
41. A. C. Kato, H. S. Katz and B. Collier, *Nature Lond.* **249**, 576 (1974).
42. W. W. Douglas and A. M. Poisner, *J. Physiol., Lond.* **183**, 236 (1966).
43. P. Banks *Biochem. J.* **101**, 536 (1966).
44. J. E. Heuser, in *Society for Neuroscience Symposia*, Vol. II, (Eds. W. M. Cowan and J. A. Ferrendelli), Bethesda, pp. 219–239 (1974).
45. E. Holtzman, *Neuroscience* **2**, 327 (1977).
46. M. P. Osborne, in *Synapses* (Eds. G. A. Cottrell and P. N. R. Usherwood), pp. 40–63. Blackie, Glasgow (1977).
47. V. A. Parsegian, in *Society for Neuroscience Symposia*, (Eds. W. M. Cowan and J. A. Ferrendelli) Vol. II, pp. 161–171. Bethesda (1977).
48. R. R. Llinas and J. E. Heuser, *Neurosci. Res. Prog. Bull.* Vol. 15, No. 4. MIT Press, Cambridge (1977).
49. R. R. Llinas, in *Society for Neuroscience Symposia*, Vol. II (Eds. W. M. Cowan and J. A. Ferrendelli), pp. 139–160. Bethesda (1977).
50. J. I. Hubbard and R. F. Schmidt, *J. Physiol. Lond.* **166**, 145 (1963).
51. L. Landmesser and G. Pilar, *J. Physiol. Lond.* **222**, 691 (1972).
52. L. Tauc, A. Hoffmann, S. Tsuji, D. H. Hinzen and L. Faille, *Nature Lond.* **250**, 496 (1974).
53. L. Tauc, in *Synapses*, (Eds. G. A. Cottrell and P. N. R. Usherwood), pp. 64–78. Blackie, Glasgow (1977).
54. L. Tauc and G. Baux, *Colloque Claude Bernard*, Paris, 5–7 Dec 1978, ed. Fondation Singer–Polignac, in press (1979).
55. B. Collier, S. Lovat, D. Ilson, L. A. Barrer and T. W. Mittag, *J. Neurochem.* **28**, 331 (1977).
56. H. Zimmerman and M. J. Dowdall, *Neuroscience* **2**, 731 (1977).
57. C. Colquhoun, W. A. Large and H. P. Rang, *J. Physiol. Lond.* **266**, 361 (1977).
58. W. A. Large and H. P. Rang, *J. Physiol., Lond.* **285**, 1 (1978).
59. W. A. Large and H. P. Rang, *J. Physiol., Lond.* **285**, 25 (1978).
60. M. J. Dennis and R. Miledi, *J. Physiol. Lond.* **239**, 571 (1974).
61. E. M. McLachlan, *Brain Res.* **98**, 373 (1975).
62. M. V. L. Bennett, P. G. Model and S. M. Highstein, in *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. XL, *The Synapse*, Cold Spring Harbor Laboratory, pp. 25–35, (1976).
63. E. Babel-Guerin, J. Boyenval, B. Droz, Y. Dunant and R. Hassig, *Brain Res.* **121**, 348 (1977).
64. M. P. Blaustein, R. W. Ratzlaff, C. Kendrick and E. S. Schweitzer, *J. gen. Physiol.* **72**, 15 (1978).
65. A. Boyne, T. P. Bohan and T. H. Williams, *J. Cell Biol.* **63**, 780 (1974).
66. A. Politoff, S. Rose and G. D. Pappas *J. Cell Biol.* **61** 818 (1974).
67. E. G. Gray and M. Paula-Barbosa, *J. Neurocytol.* **3**, 487 (1974).
68. M. P. Blaustein, R. W. Ratzlaff and S. Schweitzer, *J. gen. Physiol.* **72**, 43 (1978).
69. R. M. Marchbanks, in *Metabolic Compartmentation and Neurotransmission* (Eds. S. Berl, D. D. Clarke and D. Schneider) Plenum Press, New York, 1975.
70. R. M. Marchbanks, in *Synapses* (Eds. G. A. Cottrell and D. N. R. Usherwood), pp. 81–101. Blackie, Glasgow (1977).
71. R. M. Marchbanks, *Trends Neurosci.* **1**, 83, 168, **2**, 56 (1978).
72. B. Katz and R. Miledi, *Proc. r. Soc. Lond. Ser. B* **161**, 453 (1965).
73. J. D. Cooke and D. M. J. Quastel, *J. Physiol. Lond.* **228**, 377 (1973).
74. Y. Dunant, M. Israël, B. Lesbats and R. Manaranche, *Brain Res.* **125**, 123 (1977).
75. W. Van der Kloot, *Gen. Pharmac.* **8**, 21 1977.
76. E. G. Gray, *Int. Rev. gen. exp. Zool.* **2**, 139 (1966).
77. K. Akert, H. Moor, K. Pfenninger and C. Sandri, *Progr. Brain Res.* **31**, 223 (1969).
78. K. Akert, K. Pfenninger, C. Sandri and H. Moor, in *Structure and Function of Synapses*, (Eds. D. Pappas, and D. D. Purpura), Raven Press, New York (1972)
79. V. P. Whittaker and H. Zimmerman, *Trends Neurosci.* **1**, 100 (1978), **2**, (1979).
80. D. M. Michaelson, *Fedn. Eur. biochem. Soc. Lett.* **81**, 51 (1978).
81. J. E. Heuser, T. S. Reese, M. J. Dennis, L. Jan and L. Evans, *J. Cell Biol.* **81**, 272 (1979).
82. J. E. Heuser, *Trends Neurosci.* **1**, 80 (1978).