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APPENDIX

Morphology of Subcellular Fractions Derived from the Electric Organ of *Torpedo*

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Several studies have been carried out on the fine structure of the electric organs of torpedine fish (Luft, 1956, 1958; Wachtel *et al.*, 1961; Sheridan, 1965) and of subcellular fractions derived from them (Frontali & Toschi, 1958; Sheridan *et al.*, 1966; Israël *et al.*, 1970). However, because the conditions used to prepare the subcellular fractions studied in the accompanying paper (Whittaker *et al.*, 1972) differ significantly from those used by previous workers, a fresh investigation was made and is reported in this Appendix.

Methods

Negative staining

Vesicle preparations were fixed by the addition of 0.1 vol. of glutaraldehyde (5% in 0.4M-sucrose–0.2M-NaCl) and were applied to carbon-coated electron-microscope specimen grids by the 'double-droplet' technique (Whittaker *et al.*, 1964) for negative staining with 1% ammonium molybdate (Horne & Whittaker, 1962). Repeated applications of vesicle preparations were necessary because of the low concentration of vesicles. Spreading was promoted by coating the grids before use with 1% bovine serum albumin.

Positive staining, embedding and sectioning

Tissue fragments or suitable volumes (usually about 0.2 vol.) of suspension were placed in cold 2.5% glutaraldehyde containing 0.1M- NaH_2PO_4 – Na_2HPO_4 buffer, pH 7.4, to which had been added enough sucrose to give a final concentration of 0.2M. This buffer was approximately iso-osmotic with elasmol-

branch plasma. The tissue fragments were added to the buffered glutaraldehyde while still frozen and were allowed to thaw in the fixative. After 0.5–2 h the fixed material was collected from the glutaraldehyde by centrifugation at 5000g for 10 min; small pieces of the resultant pellet were stained with 1% OsO_4 in 0.1M-sodium phosphate buffer–0.2M-sucrose, before being dehydrated in ethanol and embedded in Araldite. Sections were cut with a diamond knife on a Sorvall Porter–Blum MT II ultramicrotome; then they were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope. Material was examined from fractions derived from both *Torpedo nobiliana* and *Torpedo marmorata*, with similar results.

Results

After being frozen and comminuted to a coarse powder, the fragments of electroplaques retain enough basic organization to be readily identifiable (Plate 1b). The innervated surface of the electroplaque, which is lined with nerve endings and subsynaptic invaginations, may be distinguished on almost every fragment. A striking feature of many of the nerve endings is the distortion, disruption and occasional absence of the nerve terminal membrane on the side away from the cleft. Parts of the endings themselves may be distorted or retracted from the electroplaque surface. The extracellular basal lamina material, which normally lies over the nerve ending surface, often appears consolidated in aggregates along the disrupted neuronal surface, mixed with remnants of the Schwann cell covering of the nerve endings. Nevertheless, numerous synaptic vesicles

are still present in the damaged terminals, where they are presumably held in place by a layer of frozen cytoplasm.

Samples of a suspension of the powdered electric organ (thawed to 0°C) in 0.2M-sucrose–0.3M-NaCl were fixed 20–30min after suspension, and when fixation was complete, the suspended fragments were collected by centrifugation (5000g for 10min). Most of the nerve endings were now seen to be depleted of their contents; many were completely empty (Plate 1c and 1d). Thawing and suspension in sucrose–saline extracted the vesicles from the broken nerve endings together, presumably, with soluble cytoplasm.

The procedure for making the cytoplasmic extract (fraction S₁₂) [described in the main paper (Whittaker *et al.*, 1972)] involves removing the coarse particles, by squeezing the suspension through muslin, brief homogenization, and removal of small tissue fragments by centrifuging in fields of intermediate intensity. This results in compact masses of tissue fragments whose morphological relationships are disrupted to a point that precludes positive identification of the emptied remains of nerve endings.

Plate (1a) shows the particulate material present in the VP peak obtained after density-gradient separation of fraction S₁₂. This consists of a fairly uniform population of vesicles of the expected size range for synaptic vesicles. The magnification of the microscope was not calibrated, so that diameters measured could err by as much as 10% from the values obtained.

Very vigorous and thorough pounding of frozen tissue, with the production of a very fine, rather than a coarse powder, resulted in a highly fragmented preparation in which organized pieces of electroplaque

cells were hard to find. Since it is difficult to standardize the comminution procedure exactly, it may be expected that varying amounts of membrane fragments will be recovered in fractions S₁₂ and MP; this was found to be so.

Why the tissue breaks up in this convenient way is not clear. It may be that long strands of fine axon terminals are ripped or peeled off the innervated surfaces of the electroplaques as the columns of cells collapse during the initial crushing and grinding of the frozen tissue. Presumably the portion of the pre-synaptic nerve terminal adjacent to the cleft remains relatively stable because of its close adhesion to the post-synaptic membrane.

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EXPLANATION OF PLATE I

Electron micrographs of subcellular fractions from electric tissue of Torpedo

(a) Peak VP [Fig. 2, main paper (Whittaker *et al.*, 1972)], negatively stained with 1% ammonium molybdate after fixation with 0.5% glutaraldehyde in 0.4M-sucrose–0.2M-NaCl, showing the presence of vesicles of mean diameter ($\pm$ S.E.M.) of 81 ± 17 nm (116) [810 ± 167 Å (116)]; (b) fragment of electroplaque cell obtained by comminuting frozen electric tissue; (c) and (d) similar fragments after suspension in 0.2M-sucrose–0.3M-NaCl for 30 min. Note: in (b) the presence of intact vesicles (V) in the nerve terminals, regions where the external prejunctional terminal membrane is disrupted or absent (filled arrows), other regions of accumulated extracellular basal lamina material covering damaged nerve ending surfaces (outlined arrow) and infoldings of the postjunctional membrane seen in oblique (F) or transverse (circled) section; and in (c) and (d) depleted nerve endings (filled arrows) partially enclosed by basal lamina, nerve plasma membrane and Schwann cell remnants, and with patent communications between the endings and extracellular space; other membranes are unaffected.