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CHOLINERGIC STIMULATION OF PHOSPHOLIPID LABELLING FROM [^{32}P]ORTHOPHOSPHATE IN GUINEA-PIG CORTEX SYNAPTOSOMES *IN VITRO*: SUBSYNAPTOSOMAL LOCALIZATION

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Abstract—Subsynaptosomal localization of stimulation of phospholipid labelling by cholinergic agents was investigated. Synaptosomes prepared from guinea-pig cortex were incubated with [^{32}P]orthophosphate in the presence or absence of 10^{-3} M carbamylcholine. Following incubation and osmotic shock, lysed synaptosomes were subjected to density gradient fractionation. Subsynaptosomal fractions were examined by electron microscopy and analysed for enzyme activities and ^{32}P-labelled lipids.

In the absence of carbamylcholine, labelled phosphatidate and phosphatidylinositol were recovered in layers and interfaces A, B, C and D formed over 0·9, 1·1, 1·2 and 1·3 M sucrose, with highest amounts of label in fractions C and D for both lipids. Carbamylcholine induced the greatest increment in these two labelled lipids in fractions A and B. This distribution correlated with the presence of acetylcholinesterase activity and membrane ghosts. No correlation was found among the four fractions between the induced increase in labelling and succinic dehydrogenase activity or with the abundance of mitochondria, synaptic vesicles, or cytoplasmic fragments identified by electron microscopy. In contrast with the increases seen in phosphatidylinositol and phosphatidate labelling, carbamylcholine caused a decrease in ^{32}P-labelling of the polyphosphoinositides, and this effect was seen primarily in the heavier subsynaptosomal fractions, C and D.

INCREASED labelling of certain phospholipids from $^{32}P_i$ in response to hormones or secretagogues has been reported for a number of different tissues *in vitro* (HOKIN, 1968). In the case of nervous tissue (excised sympathetic ganglia or brain slices) this effect has been implicated in the mechanism underlying the chemo-electrical events of synaptic transmission. Electrical stimulation of these preparations (LARRABEE *et al.*, 1963; PUMPHREY, 1969) or the addition of acetylcholine (ACh) or carbamylcholine (CCh) to incubation media (HOKIN *et al.*, 1960) increases labelling of phosphatidylinositol (PhI) and/or phosphatidic acid (phosphatidate, PhA), which is sensitive to block by inhibitors of synaptic transmission (REDMAN & HOKIN, 1964; LARRABEE & LEICHT, 1965).

We have previously demonstrated (SCHACHT & AGRANOFF, 1972; SCHACHT & AGRANOFF, 1973) effects of cholinergic agents on [^{32}P]lipid labelling in a 'light' synaptosomal fraction of guinea-pig cortex. To gain further insight into the nature and physiological significance of this effect, we have examined the subsynaptosomal distribution of radioactivity following cholinergically-stimulated lipid labelling, with concomitant biochemical and electron microscopic analyses. In these studies, CCh has been used, since we have previously demonstrated that it produces effects on lipid labelling similar to ACh (SCHACHT & AGRANOFF, 1973) and has the added advantage of not being cleaved by acetylcholinesterase (AChE).

MATERIALS AND METHODS

Preparation of synaptosomes

The methods previously described (SCHACHT & AGRANOFF, 1972) were followed with minor modifications.

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Abbreviations used: CCh, carbamylcholine; PhE, phosphatidylethanolamine; PhA, phosphatidic acid (1,2-diacyl-*sn*-glycero-3-phosphate); PhI, phosphatidylinositol; PhIP, $PhIP_2$, phosphatidylinositol phosphate, -diphosphate; SDH, succinate dehydrogenase.

Procedures were carried out at 0–4°C, unless otherwise noted. Sucrose solutions contained 1 mM sodium glycylglycinate, pH 6·6. A 10% homogenate (w/v) of cerebral cortex from albino male guinea pigs (Camm Research, Wayne, N.J.) weighing approximately 250 g was prepared in 0·32 M sucrose using a Teflon–glass homogenizer (clearance 0·19 mm) at 1000 rev/min. The homogenate was subfractionated (GRAY & WHITTAKER, 1962) into a pellet (10 min at 1000 *g*) containing unbroken cells and nuclei, and a supernatant fraction. The pellet was washed once and the combined supernatants were centrifuged for 20 min at 13,000 *g* to obtain a crude nerve ending-mitochondria pellet. This pellet was suspended in 30 ml of 0·32 M sucrose with hand homogenization, layered over three discontinuous density gradient tubes of an SW25 rotor, each containing 10 ml of 0·8 M sucrose over 10 ml of 1·1 M sucrose, and centrifuged for 2·5 h at 62,000 *g*.

The interface over 1·1 M sucrose was harvested, diluted with 0·16 M sucrose and concentrated by centrifugation at 150,000 *g* for 30 min. The pellet was resuspended by hand homogenization in 0·32 M sucrose to give the synaptosomal fraction. The average yield of nerve endings ($NE_{1.1}$) was 10 mg (protein) per g of brain (wet weight).

Incubation of synaptosomes

Incubations were performed at 37°C in air with shaking in the following medium (final concentrations): 100 mM sodium glycylglycinate, pH 6·6, 160 mM sucrose, 0·8 mM $MgSO_4$, 1 mM sodium pyruvate, 1 mM sodium fumarate, 1 mM cytidine, 1 mM *myo*-inositol and 0·1 mM NaH_2PO_4, all in 2·0 ml. Carrier-free $H_3{}^{32}PO_4$ (New England Nuclear, Boston, Mass.) in 0·02 N HCl was neutralized with an equal volume of 0·02 N NaOH.

Subsynaptosomal fractionation

At the end of the incubation period the synaptosomes were separated from the chilled (2°C) incubation medium by centrifugation for 20 min at 15,000 *g*. There was no [^{32}P]lipid in the supernatant. The pellet was suspended in 0·32 M sucrose to a volume of 1·5 ml, osmotically shocked by the addition of 8·5 ml of cold sodium glycylglycinate, pH 8·0, and kept in ice for 1·5 h (COTMAN & MATTHEWS, 1971). The incubation mixtures were then layered over a discontinuous sucrose gradient consisting of 5 ml each of 38, 35, 32.5 and 28% sucrose (w/w) and centrifuged for 1·5 h at 25,000 rev./min in a SW25 rotor. In Experiment 1 (Table 1) bands at the interfaces were collected. In Experiment 2, the bands and most of the overlayering sucrose were collected. At this point, aliquots were removed for electron microscopy. For enzymatic and lipid analyses, the fractions were diluted with 0·16 M sucrose and concentrated by centrifugation at 150,000 *g* for 30 min. Pellets were taken up in 0·16 M sucrose. Designation of fractions: A, interface between the overlying lysed incubation mixture and 28% sucrose (0·9 M); B, interface between 28 and 32·5% (0·9 and 1·1 M); C, interface between 32.5 and 35% (1·1 and 1·2 M); D, interface between 35 and 38% (1·2 and 1·3 M); E, pellet.

Electron microscopy

Aliquots of the $NE_{1.1}$ and subsynaptosomal fractions A, B, and C were diluted with 1 to 2·5 vol. of cold 2% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·2, to yield a protein concentration of approx 0·15 mg/ml. Samples were placed in cylindrical Beem capsules (Ernest Fullam, Inc., New York), prepared according to COTMAN & FLANSBURG (1970), and centrifuged at 35,000 *g* for 30 min, resulting in a disc-shaped pellet 0·3 mm thick. Fixative was replaced three times during the next hour followed by several changes of the buffer. Pellets were post-fixed for 1 h in Dalton's chrome osmium (1955) and stained *en bloc* with 0·5% aqueous uranyl acetate in 0·54% sucrose for an additional hour. Dehydration was carried out in the capsules; pellets were removed during the absolute ethanol change, further dehydrated with propylene oxide, and infiltrated with Epon. The pellet discs were flat-embedded in Epon and cured at 60°C for 72 h. Sections were cut with a diamond knife; discs were oriented such that each section provided a view of the entire thickness of the pellet. The sections were stained with ethanolic uranyl acetate and lead citrate (REYNOLDS, 1963) and examined in a Siemens Elmiskop I electron microscope at an accelerating voltage of 80 kV. Series of 5–16 micrographs were taken at successive depths through each pellet at a magnification of 10,000× and were enlarged 3× photographically for quantitative estimations.

The structural components considered in the analysis of micrographs were defined as follows:

Synaptic vesicle (*SV*). An electron lucent vesicle 500 Å dia. This category was sub-divided into SV+, a vesicle seen within an intact membrane ghost, and SV−, a vesicle not enclosed by a membrane ghost.

Synaptosome (*S*). Process containing at least two synaptic vesicles.

Mitochondrion (*M*). A structure with recognizable cristae.

Membrane fragment (*MF*). A length of membrane not part of an intact, identifiable structure.

Cytoplasmic fragment (*CF*). A membrane-bounded process containing material of cytoplasmic density.

Membrane ghost (*MG*). An intact membrane profile with a circumference of 0·785 μm or larger.

Small membrane ghost (*sm MG*). An intact membrane profile with a circumference of less than 0·785 μm.

Broken ghost (*BG*). A ghost profile broken at one or more sites.

Due to the irregular shape of the membrane ghosts in the subsynaptosomal fractions, their size was characterized on the basis of circumference, which was measured directly using a Compass 'map measure' (No. 86). Ninety-six per cent of the intact synaptosomes in the $NE_{1.1}$ fraction had a circumference of 0·785 μm or larger.

In order to obtain an estimate of the relative area of a micrograph which was occupied by each of these components, the micrographs were punctured with a 23 × 17 cm grid of 391 points, 1 cm apart. Points on or within the above components were appropriately scored. The percent area attributable to each component was calculated by dividing the number of points over that component by the

TABLE 1. STIMULATION OF $^{32}P_i$ INCORPORATION INTO SYNAPTOSOMAL PHOSPHOLIPIDS BY CARBAMYLCHOLINE

Lipid	Experiment 1 Control	Experiment 1 $+10^{-3}$ M CCh	Experiment 2 Control	Experiment 2 $+10^{-3}$ M CCh
	d.p.m. ^{32}P			
PhA	10,160	20,800	24,370	48,700
PhI	5550	10,160	9100	15,010
PhIP	21,960	18,400	22,820	17,030
$PhIP_2$	28,140	24,940	31,450	27,900

Experiment 1: $NE_{1\cdot1}$ fraction (3·8 mg protein) was incubated for 55 min with 100 μCi $^{32}P_i$. Experiment 2: $NE_{1\cdot1}$ fraction (4·0 mg protein) was incubated for 50 min with 250 μCi $^{32}P_i$. Lipids were extracted and separated as described in 'Materials and Methods'.

total number of points over all structures. The omission of points on which no components are present corrects for variability in packing density and permits direct comparison with biochemical analyses calculated as specific activities (per mg protein).

Lipid analysis

Lipids were extracted from 1 ml fractions with 4 ml chloroform–methanol-concentrated HCl–2 M KCl (1:2: 0.03 : 1; by vol.), washed (HAJRA *et al.*, 1968) and separated on TLC plates (Brinkmann Silplate 22) in two solvent systems. Solvent A, chloroform–methanol–aqueous NH_3–H_2O (45 : 45 : 4 : 11; by vol.) (GONZALES-SASTRE & FOLCH-PI, 1968) separated phosphatidylinositol phosphate (PhIP) and phosphatidylinositol diphosphate ($PhIP_2$) but not PhA and PhI; solvent B, chloroform–methanol–glacial acetic acid–H_2O (50 : 32 : 11 : 3; by vol.) gave good separation of PhA and PhI. Other major lipids were also resolved and identified by cochromatography with known standards. Radioactivity in PhA was further identified by high voltage electrophoresis after alkaline methanolysis (HAJRA *et al.*, 1968). The water-soluble product co-migrated with α-glycerophosphate. [^{32}P]-Lipids were located on TLC plates by radioautography on X-Ray film, scraped, and counted by liquid scintillation spectrometry.

Other assays

AChE (EC 3.1.1.7) was assayed at 25 C by the method of ELLMAN *et al.* (1961), SDH (succinate-INT-reductase, EC 1.3.99.1) according to ELAM & AGRANOFF (1971) and protein was determined by the method of LOWRY *et al.* (1951). Reagents were purchased as described previously.

RESULTS

Incubation and lysis of synaptosomes

As previously demonstrated (SCHACHT & AGRANOFF, 1973), CCh stimulated $^{32}P_i$ incorporation into PhA and PhI by 100 and 70 per cent respectively and decreased labelling of PhIP and $PhIP_2$ by 15–20% (Table 1). Labelling of PhE the only other significantly labelled lipid (SCHACHT & AGRANOFF, 1972) was an order of magnitude lower and was not studied further, since PhE labelling is known to be unaffected by CCh (Table 2). There was an approximately 20 per cent loss of [^{32}P]-lipid as a result of the lysis, but the per cent distribution of the labelled lipids remained essentially unchanged (Table 2).

Subsynaptosomal fractionation

Two-thirds or three-fourths (Table 1, Exps. 1 and 2 respectively) of the incubation mixture were employed for the subsynaptosomal fractionation on sucrose gradients. The three SW25 rotor buckets contained the lysed $NE_{1\cdot1}$ fraction following incubation with and without CCh, and an incubation without $^{32}P_i$ for enzymatic and electron microscopic analyses. Recoveries were lower in Exp. 1, where only the visible bands at the interfaces were harvested; in Exp. 2, most of the overlying sucrose above each interface was also collected. Recoveries were: protein, 55 per cent (Exp. 2, 72 per cent); AChE, 50 per cent (65 per cent); SDH, 52 per cent (61 per cent); PhA and PhI (with

TABLE 2. DISTRIBUTION OF ^{32}P AMONG PHOSPHOLIPIDS BEFORE AND AFTER LYSIS OF SYNAPTOSOMES

	Per cent of total ^{32}P lipid					
	PhA	PhI	PhIP	$PhIP_2$	PhE	Others*
Control before lysis	9·1	5·0	27·8	31·9	1·1	25·1
Control after lysis	9·4	6·1	27·7	31·2	1·4	24·2
+CCh before lysis	17·8	8·3	21·7	24·7	1·0	26·5
+CCh after lysis	17·2	10·4	22·1	25·1	1·5	23·7

* Unidentified materials mainly remaining at origin or travelling with solvent front.
Conditions were those of Exp. 2, Table 1.

and without CCh) 44–50 per cent (80–87 per cent); PhIP and $PhIP_2$ (with and without CCh) 35–40 per cent (70–80 per cent).

Protein distribution is given in the legend to Fig. 1. The pellet *E* in both experiments contained < 0·1 mg protein, no significantly labelled lipids and measurements of enzyme activities showed marginal activity only. It was therefore not studied further. Similarly, the supernatant incubation mixture over 0·9 M sucrose lacked significant enzyme activities and labelled lipids. For the other fractions, the pattern of basal labelling (Fig. 1A) is very similar for PhA and PhI with more labelling in the heavier fractions, C and D. PhIP and $PhIP_2$ labelling appears more prominent in the lightest fraction, A. The distribution of the CCh-stimulated labelling does not follow the distribution of basal labelling. For PhA and PhI, the incremental incorporation is highest in fraction A, declining through the gradient with fraction D representing less than 10 per cent of the total. In fraction A, we find an enrichment of the CCh-effect. Labelling of PhA and PhI is stimulated 150 and 125 per cent respectively as compared to 100 and 75 per cent in the original $NE_{1\cdot1}$ fraction. The decrement of PhIP and $PhIP_2$ label appears most pronounced in the heavier fractions, C and D.

AChE activity (Fig. 1B) is greatest in fraction A and declines through the gradient, with fraction D representing approximately 10 per cent of the total. SDH is most active in fraction C, with significant but lesser amounts in B and D.[1]

Electron microscopy

Stratification was seen throughout each pellet, confirming the necessity for a method of specimen preparation that allows for systematic examination of the entire thickness of the pellet (COTMAN & FLANSBURG, 1970). In fraction C, for example, free synaptic vesicles occupied 1·5 per cent and membrane ghosts 32 per cent of the total structure area analysed through one-half of the pellet; in micrographs from the other half, free vesicles accounted for 11 per cent and ghosts, 18 per cent of the total structure area. The relative area occupied by membrane ghosts of different sizes shifted depending on the depth of the pellet being analysed. At one edge of pellet C, ghosts with a circumference between 1·57 and 1·88 μm occupied 11 per cent of the relative area; at the other edge, ghosts of this circumference occupied only 1 per cent. Although the relative area accounted for by membrane ghosts decreased from fraction A to fraction C, larger ghosts accounted for a greater proportion of the total ghost area in fraction C. For each pellet, the values at the various depths were combined to give an average distribution of structures in the gradient fraction from which it was derived.

The per cent structure areas occupied by various components of the $NE_{1\cdot1}$ fraction (Fig. 2) were as follows: synaptosomes account for 14 per cent of the area; broken membrane ghosts, 14 per cent; membrane ghosts, 24 per cent; small membrane ghosts, 14 per cent; membrane fragments, 11 per cent; cytoplasmic fragments, 22 per cent; unenclosed synaptic vesicles, <1 per cent; free mitochondria, < 1 per cent; total mitochondria, including those within synaptosomes and cytoplasmic particles, 4 per cent.

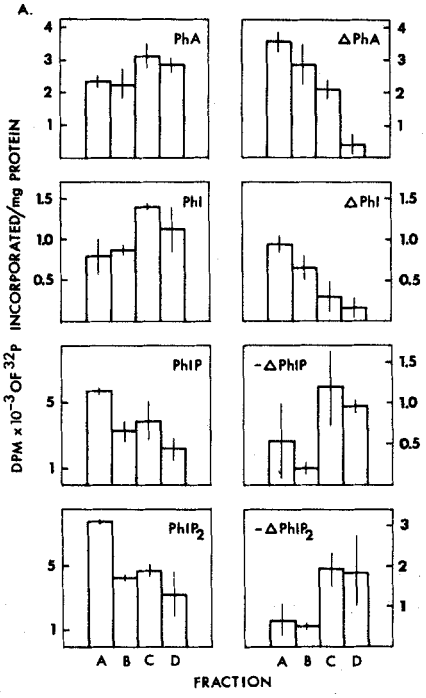

FIG. 1A. Subsynaptosomal distribution of [^{32}P]-lipids. The figures represent the averages of two experiments (see Table 1) with vertical bars indicating the range of duplicates. Incorporation was calculated per mg protein per 100 μCi $^{32}P_i$. Left column is basal labelling without CCh, right column is the CCh-induced incremental incorporation. Note that Δ is negative for PhIP and $PhIP_2$. Protein distribution among subfractions: A, 0·20 mg (Exp. 2, 0·36 mg); B, 0·49 mg (0·75 mg); C, 0·40 mg (0·60 mg); D, 0·21 mg (0·32 mg).

[1] In a third experiment, several months later in which an SW27 rotor was used for synaptosomal and subsynaptosomal fractionation, fraction B had an approximately 10% higher specific activity of AChE than fraction A; the incremental labelling of PhA and PhI were similarly higher in B.

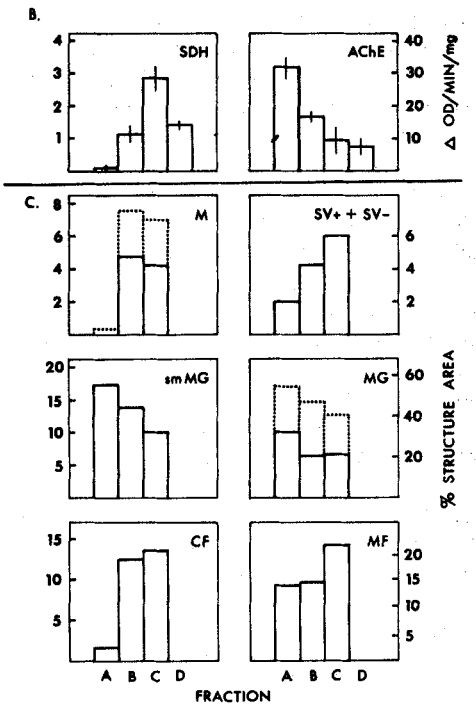

FIG. 1B. Subsynaptosomal distribution of AChE and SDH. Figures for AChE and SDH represent the average of two experiments. See legend to Fig. 1A.

FIG. 1C. Plots of distribution of identifiable subcellular elements by electron microscopy. M, mitochondria and structures presumed to be extruded cristae (broken lines); SV, the sum of synaptic vesicles within intact membrane ghosts (SV+) and unenclosed synaptic vesicles (SV−); smMG, small membrane ghosts; MG, membrane ghosts and broken ghosts (broken lines); CF, cytoplasmic fragments; MF, membrane fragments. Areas surveyed were 255 μm^2 (fraction A); 216 μm^2 (fraction B); and 255 μm^2 (fraction C). Structural components occupied approximately 65% of these areas. Fraction D was not examined.

Histograms of the area occupied on micrographs by the structural components of subsynaptosomal fractions A, B and C, relative to the total area of all structures in each fraction, are presented in Fig. 1C. Figures 3, 4 and 5 are electron micrographs of pellets prepared from fractions A, B, and C respectively. Recognizable mitochondria were present in fractions B and C, and were not seen in A. Densely staining tubular structures, possibly representing condensed cristae extruded from lysed mitochondria, were also found in fractions B and C and were rarely seen in fraction A. Synaptic vesicles occur in all three fractions, being most numerous in fraction C. Myelin fragments were only occasionally found in B and C, being most concentrated at one edge of A. Points over and enclosed by myelin accounted for 12 per cent of the structure area in A.

DISCUSSION

The stimulation of phospholipid labelling by neurohumors or neurotransmitters is well-documented for a number of neural tissues, including brain cortex and sympathetic ganglia (HOKIN, 1968). The precise intracellular location of these effects remains unknown. In the case of cholinergic stimulation we can consider three possibilities: A, a postsynaptic site, possibly secondary to neurotransmitter interaction with a cholinergic receptor; B, a presynaptic site related either to ACh release and/or exocytosis of synaptic vesicles or to uptake of ACh and/or endocytosis; C, both pre- and postsynaptic sites, perhaps a diffuse tissue distribution.

The postsynaptic argument is based primarily on the observation that the enhanced labelling is elicited experimentally by addition of neurotransmitter to the neural preparations, such as brain slices or intact ganglia, and that blockers of cholinergic transmission block the effect (LARRABEE & LEICHT, 1965). Further, addition of ACh produces the characteristic stimulation of $^{32}P_i$ labelling of PhA and PhI in isolated sympathetic ganglia, even after denervation and degeneration of the presynaptic elements (HOKIN, 1966). Additional support for a postsynaptic site of the effect comes from the reported isolation of ACh-stimulated [^{32}P]-PhI in a subcellular fraction thought to be rich in cholinergic receptors in rat brain (LUNT *et al.*, 1971), although this has been disputed (LAPETINA & MICHELL, 1972). Various arguments for the postsynaptic site of the ACh effect have been summarized by DURELL *et al.* (1969) who suggested that diglyceride availability in the postsynaptic membrane is rate-limiting for ^{32}P incorporation into lipid. It was proposed that PhI phosphodiesterase or polyphosphoinositide phosphodiesterase mediates the effect via ACh-directed diglyceride release. Using techniques which permitted extraction and identification of labelled PhIP and $PhIP_2$, we found a decrease in labelling in these lipids in the presence of added cholinergic agents. However, the decrease in labelled polyphosphoinositides does not appear to be specific to the addition of cholinergic agents, since it is not blocked by cholinergic inhibitors, nor is it localized in the 'light' synaptosomal fraction (SCHACHT & AGRANOFF, 1972; SCHACHT & AGRANOFF, 1973). As shown further in the present study, the decrease in polyphosphoinositide labelling is not localized in subsynaptosomal fractions that show the maximal stimulation of [^{32}P]-PhA and -PhI formation.

In addition to the questions concerning the involvement of specific enzymes in the postsynaptic membrane, the question is raised whether a postsynaptic site is at all involved in the stimulation of labelling. It has been argued, for example, that while the increased [^{32}P]-lipid labelling may be in the postsynaptic neuron, it may not be confined to the synaptic region. Radioautographic studies of [^{3}H]inositol incorporation in sympathetic ganglia led to the proposal that the stimulation of labelling is in the endoplasmic reticulum, and is perhaps related to protein transport (HOKIN, 1965). Our finding of a cholinergic lipid labelling effect in the nerve ending-mitochondrial fraction of the crude homogenate of brain (SCHACHT & AGRANOFF, 1971) indicates that for ^{32}P-labelling in the preparation, the stimulation is localized either to the presynaptic region or to the attached postsynaptic structures of synaptosomes. While this finding would appear to be in conflict with the radioautographic studies cited above, it remains possible that the difference may be related to the use of different isotopic precursors and tissue preparations in the various experiments. Antidromic stimulation of intact ganglia does not produce increased ^{32}P incorporation into lipids, suggesting that the formation of ^{32}P-labelled PhA and PhI, if postsynaptic, is not related to events following depolarization of the postsynaptic membrane. It is alternatively possible that the antidromic impulses failed to invade the soma (LARRABEE & LEICHT, 1965).

A principal reason for reconsidering the possibility that the ^{32}P-stimulated labelling of PhA and PhI is presynaptic derives from recent studies which localize the cholinergic effect in a synaptosomal fraction of brain (SCHACHT & AGRANOFF, 1972; YAGIHARA & HAWTHORNE, 1972), although a significant contribution by adhering postsynaptic membranes cannot be excluded. Of possible presynaptic mechanisms, it seems unlikely that the effect is related to release of neurotransmitter or exocytosis of synaptic vesicles, since the stimulation of ^{32}P incorporation is not affected by addition of calcium ion nor inhibited by the addition of EDTA or magnesium ion (SCHACHT & AGRANOFF, 1972; YAGIHARA *et al.*, 1973). A more appealing prospect is that cholinergic stimulation is involved in reuptake of neurotransmitter and/or endocytosis. ACh addition is known to stimulate its uptake into brain slices, and atropine has been shown in several instances to block this process (POLAK & MEEUWS, 1966; SCHUBERTH & SUNDWALL, 1967) as well as the uptake of CCh (CREESE & TAYLOR, 1965). Reuptake of ACh has, however, been reported to be blocked by tubocurare in rat brain slices (LIANG & QUASTEL, 1969), yet we find this drug at 10^{-4} M has no effect on the [^{32}P]-PhA and PhI stimulation in the guinea-pig synaptosomal preparation (SCHACHT & AGRANOFF, 1972). While this latter finding might argue against a relationship between the ACh effect on lipid labelling and its uptake, it remains possible that the ^{32}P-labelling effect is confined to a population of central muscarinic cholinergic receptors that represent a small fraction of the synaptosomes whereas mediation of ACh uptake occurs mainly via nicotinic receptors, which can be blocked by tubocurare. A further question regarding the association of uptake and the ACh effect arises from the report that eserine can decrease ACh uptake in brain slices (SCHUBERTH & SUNDWALL, 1967; LIANG & QUASTEL, 1969), since the latter substance is routinely added to our synaptosomal incubations in which ACh stimulates lipid labelling.

In the present study the concentration of ACh and CCh used were those that produced the maximal stimulation of PhA and PhI labelling, although measurable stimulation was observed after addition of 10^{-6} M ACh or 10^{-5} M CCh (SCHACHT & AGRANOFF, 1972). Similar results have been reported in synaptosomal preparations (YAGIHARA & HAWTHORNE, 1972) and in brain homogenates (REDMAN & HOKIN, 1964). The ionic composition and added energy source of the incubation medium used were those that were found to support a maximal and reproducible lipid labelling effect, rather than to reproduce what one might guess would be a physiological milieu for isolated synaptosomes (SCHACHT & AGRANOFF, 1972).

The morphological analysis of the synaptosomal fraction $NE_{1\cdot1}$ (Fig. 2) is not unlike that reported by GAMBETTI *et al.* (1972) for a synaptosomal fraction isolated in discontinuous Ficoll gradients from cortices of 18-day-old rats (AUTILIO *et al.*, 1968). Extensive analysis of this fraction showed that processes of 0·3 μm dia containing at least three synaptic vesicles occupied approximately 23 per cent of the structure area and accounted for 74 per cent of the identifiable particles. COTMAN & MATTHEWS (1971) have reported a similar (60–75 per cent) synaptosome count for a rat cerebral cortex synaptosomal fraction isolated from a Ficoll-sucrose gradient. The technique of lysis and subfractionation employed here was reported by COTMAN & MATTHEWS (1971) to be superior in terms of resolution of membrane and mitochondrial fractions. Since this method includes a 1·5 h lysis at 0°C, the question arises whether a transfer of lipids between subcellular structures (MILLER & DAWSON, 1972) could occur during this time. This possibility is unlikely since the reported exchange requires the presence of a macromolecular component contained in the brain supernatant fraction, and is relatively slow for synaptosomal fractions, even at 37°C.

Comparison of the electron micrographs of the

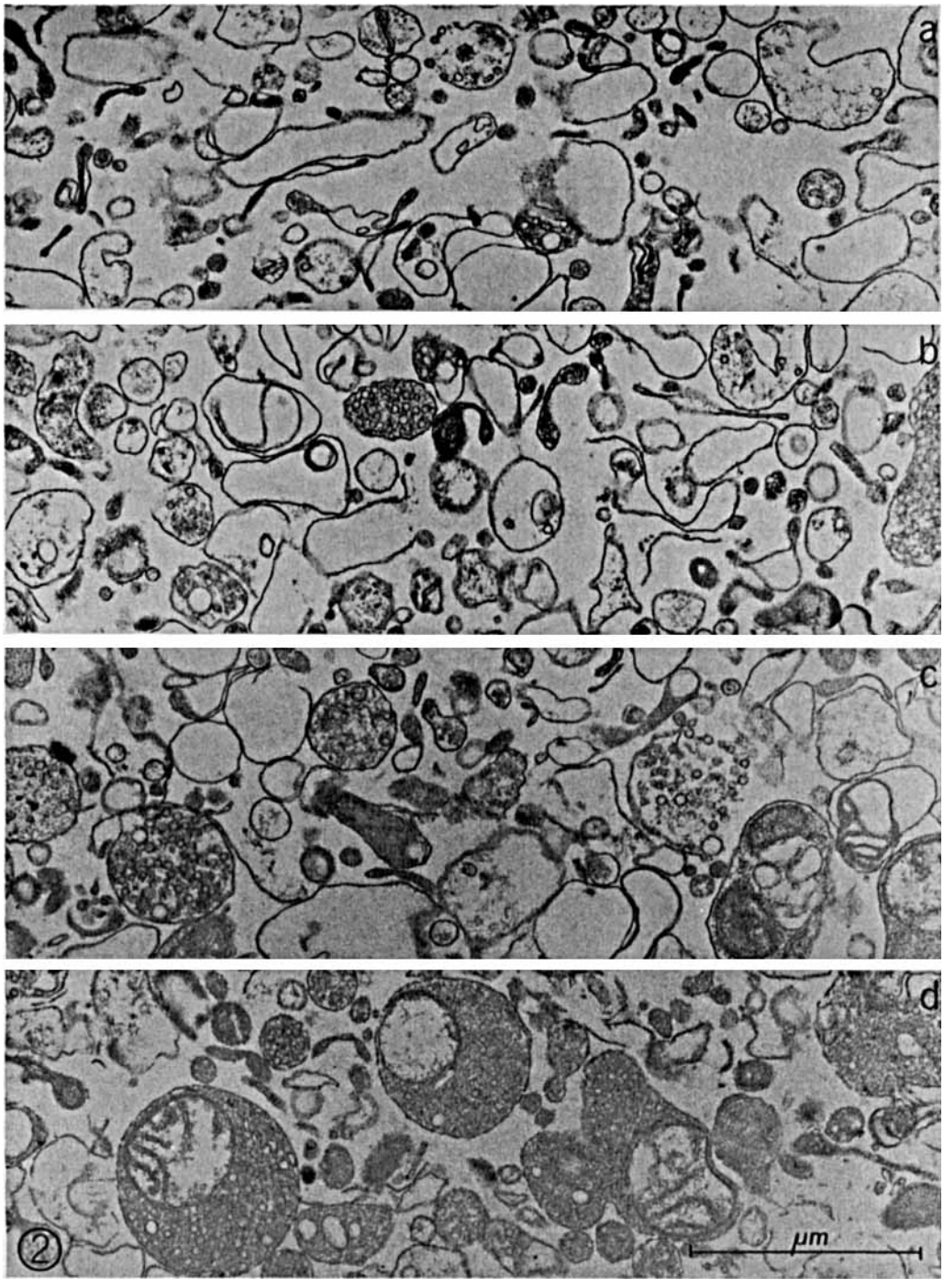

FIG. 2. Electron micrographs taken through the middle two-thirds of fraction NE1·1 before lysis. Numerous intact synaptosomes are found in the deeper portions (C and D) of the pellet. ×30,000.

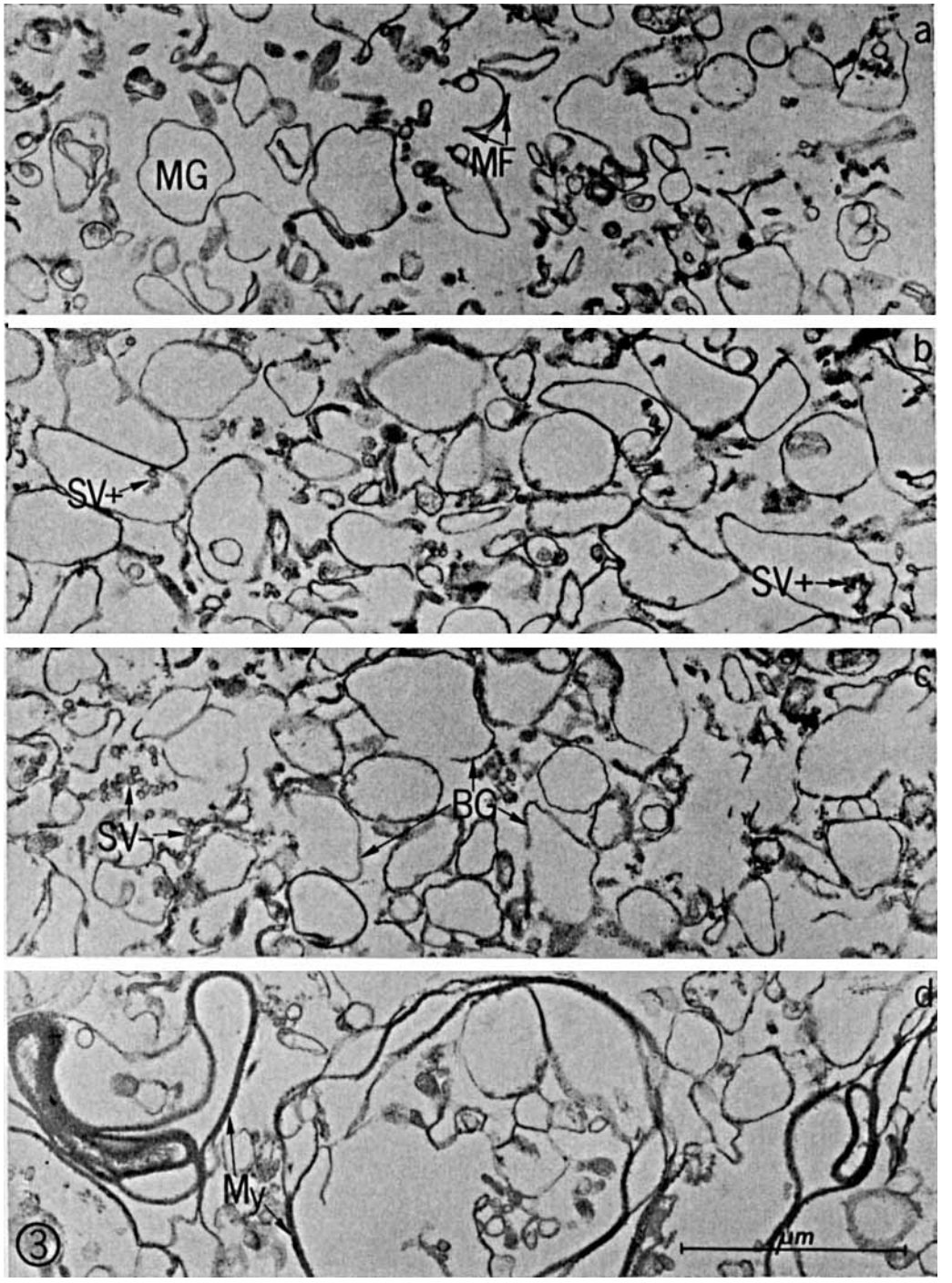

FIG. 3. Electron micrographs taken at the edges (a and d) and from the middle regions (b and c) of a pellet prepared from subsynaptosomal fraction A. Membrane profiles are prominent structures in this fraction. Myelin is concentrated at one edge of the pellet. See Methods for abbreviations. ×30,000.

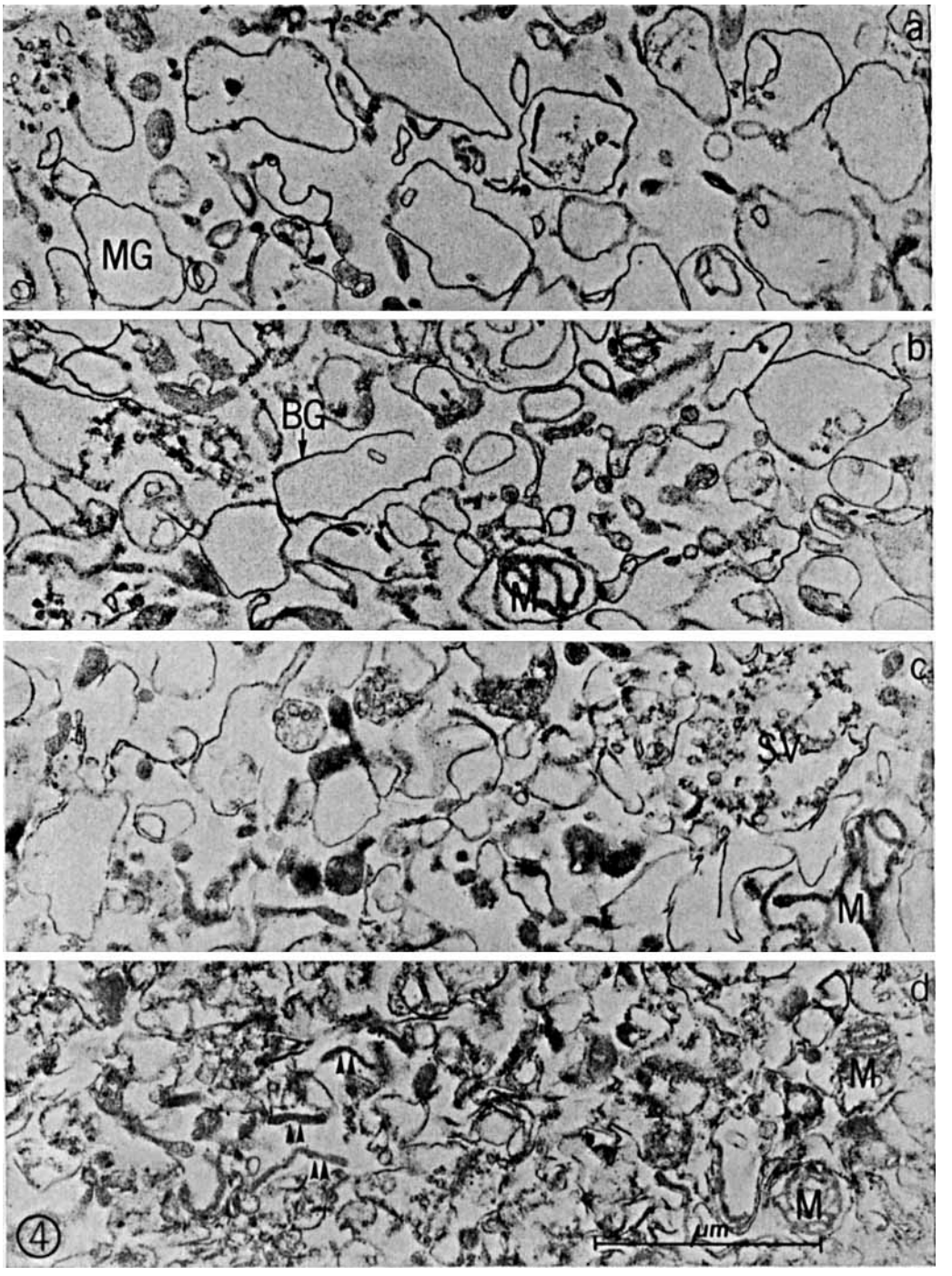

FIG. 4. Electron micrographs through a pellet prepared from subsynaptosomal fraction B. Arrowheads, densely staining tubular structures resembling condensed cristae extruded from lysed mitochondria. ×30,000.

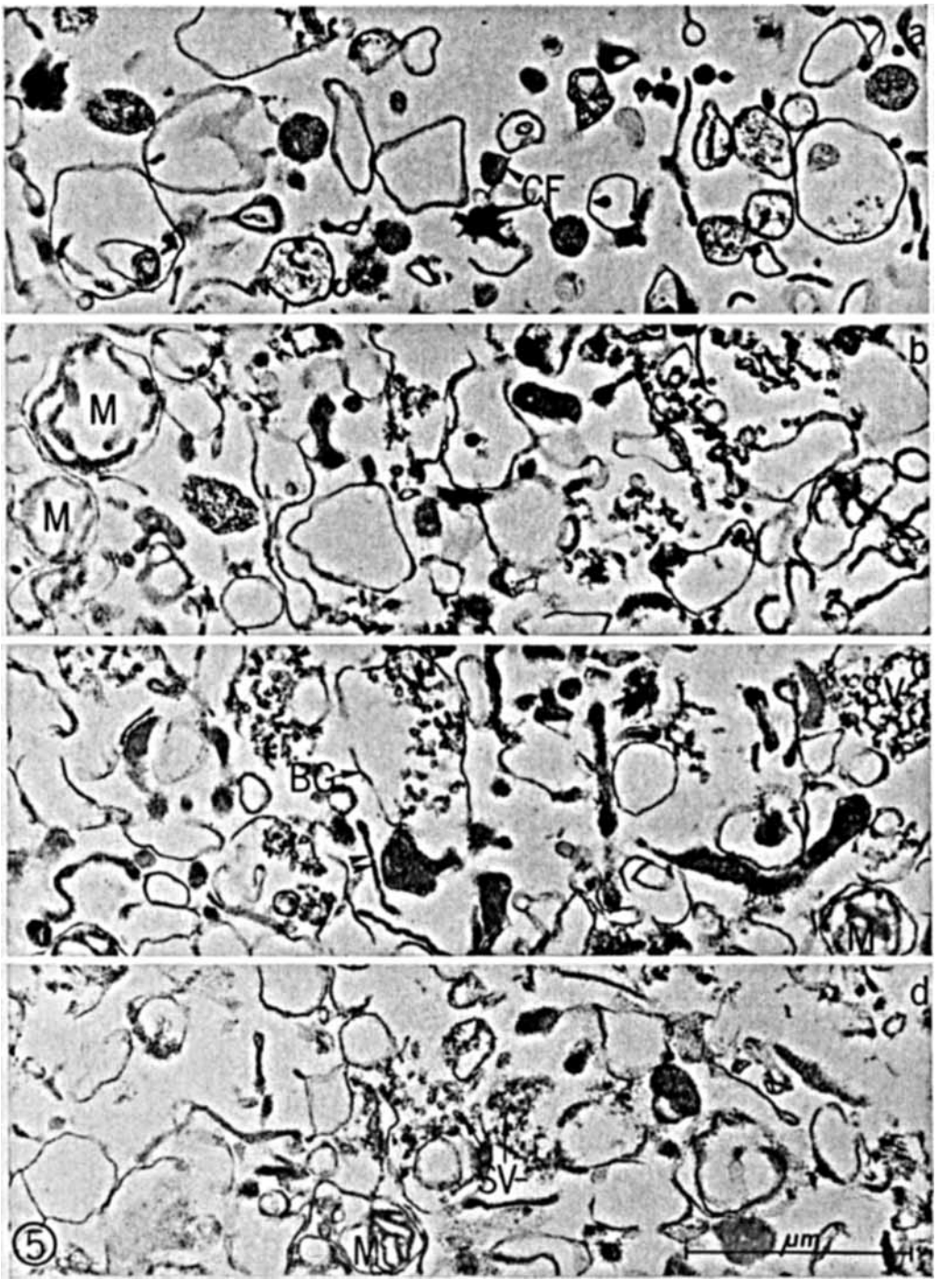

FIG. 5. Electron micrographs through a pellet prepared from subsynaptosomal fraction C. Mitochondria and synaptic vesicles are seen frequently in this fraction. Arrowheads, structures thought to be extruded mitochondrial cristae. ×30,000.

synaptosomal fraction $NE_{1\cdot1}$ (Fig. 2) with those of the osmotically-shocked fractions (Figs. 3–5) clearly shows that lysis has taken place. No intact synaptosomes remain. Intact preparations of fraction $NE_{1\cdot1}$ subjected to density gradient separation, contained 10 per cent of the total protein in fraction A, whereas after lysis, fraction A contained 15–20 per cent. Of greater significance, without prior lysis, only 1 per cent of the labelled lipid was present in fraction A whereas after lysis this fraction accounted for approximately 25 per cent of the total recovered [^{32}P]-lipids. After lysis, fraction A showed both the highest CCh-stimulated labelled PhA and PhI increment and had the highest concentration of membrane ghosts. Since the membrane ghosts in fraction A appear to be generated by lysis, and since their size is similar to that seen in the intact synaptosomes, the result is compatible with the interpretation that a significant proportion of them are derived from synaptosomes. The distribution of membrane ghosts parallels the AChE activity and is distinctly different from the subfractionation pattern of all other identifiable structures; SDH, identifiable mitochondria and synaptic vesicles are virtually absent in fraction A.

Basal labelling (in the absence of CCh) of PhA and PhI is somewhat higher in the heavier fractions whereas the reverse appears to be the case for PhIP and $PhIP_2$. The incremental ^{32}P incorporation for the two groups of lipids in the presence of CCh is also distinctive; the PhA and PhI increment is highest in fractions A and B; the PhIP and $PhIP_2$ *decrement* is most pronounced in fractions C and D. As we found with synaptosomal fractions, these subsynaptosomal experiments further distinguish the stimulation of PhA and PhI labelling by cholinergic agents from the nonspecific decrease in polyphosphoinositide labelling (SCHACHT & AGRANOFF, 1973). It is not likely that changes in the specific activity of ATP mediate either the increased or the decreased labelling (SCHACHT & AGRANOFF, 1974). From Fig. 1 we find an obvious correlation between the incremental labelling of PhA and PhI with AChE activity and with the presence of membrane ghosts in the fractions. The effect is not positively correlated with any other structure. Correlation analysis (EDWARDS, 1959) of the increments in PhA and PhI indicated what was apparent to the eye, namely good correspondence of the ΔPhA and ΔPhI with AChE (0·832, $P = 0{\cdot}005$ and 0·944, $P < 0{\cdot}005$), with small membrane ghosts (0·999, $P < 0{\cdot}01$ and 1·000, $P < 0{\cdot}01$) and with membrane ghosts (0·986, $P = 0{\cdot}05$ and 0·976, $P < 0{\cdot}05$). The correlation was negative with SDH and other structures such as synaptic vesicles, mitochondria or membrane fragments. While relatively few synaptic complexes (40) or putative postsynaptic membranes (164) were seen, both were virtually absent from fraction A, and highest in fraction C. Thus no experimental support can be inferred from these data for a role of the postsynaptic membranes in the synaptosomal lipid labelling stimulation. While we do not see a correlation with postsynaptic elements, these structures have been reported to be rich in AChE in brain (MCBRIDE & COHEN, 1972). Our study does not indicate a correlation of the stimulation of lipid labelling with the presence of synaptic vesicles. This is not surprising since it has been shown that ACh added to synaptosomes *in vitro* is incorporated into a cytoplasmic compartment but not into synaptic vesicles (MARCHBANKS, 1968). However, if the stimulated labelling is associated with uptake of ACh, it may be that a subpopulation of these organelles, such as the complex (coated) vesicles seen in intact brain (GRAY & WILLIS, 1970) are the primary site of the effect. Possibly the extensive handling of tissue in these experiments prior to electron microscopy, i.e. synaptosomal isolation, incubation, lysis and refractionation has obscured structures associated with the stimulated labelling that are ordinarily discernible. While the present experimental findings suggest a relationship between ACh transport and stimulation of PhA labelling, the nature of the interactions remains uncertain, particularly in view of the lack of independent evidence for specific reuptake of ACh.

YAGIHARA *et al.* (1973) have recently examined subsynaptosomal fractions of guinea pig cortex following incubation in the presence or absence of ACh. Differences in the details of the incubation, lysis and subsynaptosomal fractionation preclude direct comparison with the present results. In their study, the maximum [^{32}P]-PhA stimulation is localized in a fraction low in AChE, however in agreement with our findings, the authors found no effects of added calcium (SCHACHT & AGRANOFF, 1972) or EDTA. Although there were no ultrastructural studies, by comparison with the previous studies of WHITTAKER *et al.* (1964), they attribute the effect to the presence of synaptic vesicles, even though this fraction must be impure as evidenced by the reported presence of AChE, believed absent from synaptic vesicles (WHITTAKER *et al.*, 1964). Whatever the ultimate identity of the subsynaptosomal elements in the fraction, it is interesting that the amount of PhA is reported to be reduced by 60 per cent by the addition of ACh in the studies of YAGIHARA *et al.* (1973). This supports our proposal that ACh addition stimulates hydrolysis of PhA in the synaptosome (SCHACHT & AGRANOFF, 1973). Our present finding that CCh-stimulation of PhA labelling is associated with a fraction rich in membrane ghosts

is of further interest, since PhA phosphohydrolase activity has its highest specific activity in synaptosomal plasma membranes (COTMAN *et al.*, 1971).

Note added in proof. In an attempt to resolve the difference in distribution of the Ach-stimulated labelling seen in our subsynaptosomal fractions with the results of YAGIHARA *et al.* (1973), two experiments were performed with the more shallow gradient used by these authors. We did not observe maximal Ach-stimulation of labelling in the putative synaptic vesicle fraction, and again found good agreement between distribution of the incremental labelling and AchE distribution among fractions.

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