MARCAJE DE CARGAS ELECTRICAS DE SUPERFICIE EN LA MEMBRANA SINAPTOSOMAL. ESTUDIO BIOQUIMICO Y CITOQUIMICO DE ALTA RESOLUCION.

POR

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Estudios de electroforesis de partículas han demostrado que los sinaptosomas tienen una carga eléctrica neta de superficie negativa, por lo que dichas partículas se dirigen al polo positivo. Estudios correlativos de tipo bioquímico han mostrado que estas cargas de superficie están en su mayor parte conferidas por grupos carboxilo de ácido siálico y que seguramente otros elementos cargados electronegativamente contribuyen a dicha carga electronegativa. No existen a la fecha estudios citoquímicos ultraestructurales que demuestren la distribución de las cargas negativas de superficie en los sinaptosomas ni datos que hablen sobre la distribución de cargas electropositivas en la superficie de dichos sinaptosomas. Bittiger y Schnebli al utilizar ricina y concanavalina A marcadas con ferritina mostraron que la distribución de grupos supuestamente conteniendo galactosa y otros conteniendo glucosa o manosa tenían una distribución homogénea en la superficie de los sinaptosomas y se concentraban fundamentalmente en la región intersináptica.

Esto último se observó particularmente en los experimentos en que se empleó concanavalina A. Resulta difí-

cil saber si la localización de esos grupos en base a estos experimentos corresponde a la localización *in vivo* de los residuos de carbohidratos o resulta del desplazamiento de las partículas de superficie sinaptosomales inducida por la lectina empleada como es el caso observado en el fenómeno de acumulación inducida de partículas ("capping") en otras células.

En el presente trabajo se estudió el patrón de distribución de cargas eléctricas de superficie en la membrana sinaptosomal por métodos citoquímicos de alta resolución al emplear hierro coloidal a diferentes pHs y se correlacionaron estos resultados con datos obtenidos de estudios bioquímicos.

El empleo de una solución de hierro coloidal, dependiendo el pH a que se utilice, permite marcar las cargas eléctricas de superficie negativas (al emplear soluciones a pH bajo: 1.8) o cargas positivas (pH alto: 4.5). Esto parece estar basado en parte en la interacción electrostática de los grupos cargados de los elementos tisulares con la carga de las partículas coloidales de óxido de hierro.

En el presente trabajo, al utilizar soluciones de hierro coloidal a diferentes pHs y examinando las muestras con el microscopio electrónico se pudo visualizar un patrón peculiar de marcaje de cargas electronegativas y electropositivas en la superficie de los sinaptosomas. Se emplearon ratones albinos adultos, de los cuales se obtuvieron los cerebros después de decapitación, se excluyó cerebelo y se separó la fracción sinaptosomal por gradiente discontinuo de sacarosa a partir de la fracción mitocondrial cruda del homogenado cerebral. Esta fracción sinaptosomal se incubó con solución dializada de hierro coloidal a un pH de 1.8 o a un pH de 4.0 para marcar cargas de superficie negativas o positivas respectivamente. Los estudios bioquímicos realizados con fines de identificación de los elementos que contribuyen a la presencia de cargas electronegativas y electropositivas de superficie fueron digestiones enzimáticas con neuraminidasa purificada de Clostridium perfringens, identificación de ácido siálico con el método espectrofotométrico de Warren y titulación de proteínas totales en la fracción sinaptosomal.

Con el microscopio electrónico se observó un patrón

homogéneo de precipitado fino en la superficie de los sinaptosomas cuando se empleó la solución de hierro coloidal cargada positivamente. Después de la metilación de la fracción sinaptosomal o la incubación de esta fracción con neuraminidasa purificada y posterior incubación de las muestras con hierro coloidal cargado positivamente, no se observó precipitado electrodenso en la superficie de los sinaptosomas. Aproximadamente el 20% del ácido siálico total en la fracción sinaptosomal fué liberado por digestión con neuraminidasa en el presente trabajo, lo cual está de acuerdo con los resultados obtenidos por otros autores en que hacen un estudio correlativo bioquímico-electroforético. Fué insignificante la cantidad de ácido siálico liberado cuando se utilizó neuraminidasa herbida antes de la incubación de los sinaptosomas con la solución positiva de hierro coloidal. En estos experimentos se observó con el microscopio electrónico el precipitado fino homogéneamente distribuido en la superficie de los sinaptosomas.

Con la solución negativa de hierro coloidal se identificó un patrón irregular y heterogéneo en la superficie de los sinaptosomas. Este patrón de marcaje no se ob-

servó cuando se llevó al cabo una reacción de desaminación antes de la incubación con la solución negativa de hierro coloidal. La incubación con neuraminidasa purificada antes de la incubación con la solución negativa de hierro coloidal o el tratamiento con neuraminidasa seguido por desaminación e incubación con la solución negativa de hierro coloidal no modificó el patrón de marcaje en la superficie de los sinaptosomas.

Con estos datos se concluye lo siguiente:

1. En el presente trabajo se realiza por primera vez un estudio correlativo de citoquímica de alta resolución para identificación de cargas eléctricas de superficie en sinaptosomas con estudio correlativo bioquímico para la identificación de los grupos que determinan estas cargas de superficie.

2. Con el empleo de las soluciones de hierro coloidal a diferentes pHs es posible identificar el patrón de distribución de cargas en la superficie de partículas subcelulares.

3. Existe un patrón homogéneo de distribución de cargas electronegativas de superficie en el glucocálix de los sinaptosomas, en tanto que las cargas electropositivas tienen una distribución heterogénea en la superficie de los mismos.

4. Las cargas electropositivas identificadas en ese trabajo con la solución negativa de hierro coloidal probablemente corresponden a grupos amino expuestos de los componentes del glucocálix de los sinaptosomas, fundamentalmente en base a los estudios de desaminación realizados en este trabajo.

5. En base a los estudios de metilación y digestión con neuraminidasa purificada es muy probable que las cargas eléctricas negativas de superficie son conferidas por los grupos carboxilo de ácido siálico expuesto a la actividad de la neuraminidasa.

Es muy probable que este ácido siálico forme parte de gangliosidos y de glucoproteínas presentes en la cubierta exterior de las terminales sinápticas y así

constituya uno de los elementos importantes involucrados en los procesos de transmisión sináptica, como ha sido sugerido por varios autores.

LABELING OF ELECTRICAL SURFACE CHARGES AT SYNAPTOSOME MEMBRANE. Electron cytochemical and biochemical study*

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S U M M A R Y

A distinctive pattern of electronegative and electropositive charges could be visualized at the synaptosome surface coat by using colloidal iron solutions at different pH and examining the samples with the electron microscope. Synaptosome fraction from mice brains were incubated with dialized colloidal iron at pH 1.8 (Fe(+)) and at pH 4.0 (Fe(-)) for labeling surface negative and positive charges, respectively. At ultrastructural level, a homogeneous fine precipitate was seen at synaptosome surface when the Fe(+) solution was employed. No deposits were observed when methylation or incubation with purified neuraminidase were carried out prior to incubation with the Fe(+) solution.

Approximately 20% of the total synaptosome sialic acid was released by neuraminidase digestion in the present work. No significant amount of sialic acid was released when boiled neuraminidase was employed, and the deposits were present in an homogeneous pattern

at synaptosome surface. With the Fe(-) solution an irregular labeling pattern was seen at synaptosome surface. These scarce deposits were not observed when deamination reaction was performed prior to incubation with the Fe(-) solution. It is concluded that the surface electronegative charges labeled with the Fe(+) solution most likely correspond to carboxyl groups of sialic acid, whereas the deposits observed with the Fe(-) solution probably corresponds to exposed amino groups of the synaptosome surface coat components.

I N T R O D U C T I O N

The surface coat or glycocalix plays an important role in cell physiology, particularly related to cell aggregation[44] and contact recognition[24]. Glycoproteins are the essential components of surface coat in most of the cells, including the neurons, whose membranes are rich in sialoglycoproteins and gangliosides[10,21]. On the other hand, various functional characteristics of the surface coat depend upon the presence of sialic acid in its elements. In this regard, a) due to its electronegative charge, sialic acid contributes to form a hydrophilic environment at the surface cell area[13,52]; b) functioning as an ionic exchange element, sialic acid participates in the regulation of flow of cations across the plasma membrane[28,29]; c) in some cells sialic acid is an important element in cell antigenicity[16,35,37]; d) it is one of the factors implicated in intercellular adhesivity[27,39,61]; and e) in some cells, sialic acid is an important element in membrane receptors[14,15].

In the central nervous system, the neuronal plasma membrane, its surface coat and the elements present in the extracellular space act together in the regulation of ionic transport involved in membrane excitation and recovery[29]. Electrophoretic mobility of brain synaptosomes depends upon their negative surface charges[58], conferred either by sialic acid residues, which are present in sialoglycoproteins and gangliosides in nerve tissue elements[18,22,29,47,50,59], or by acid mucopolysaccharides present in the synaptosome membrane[12,57].

Although some controversy exists as to the participation of sialic acid in the synaptosome electrophoretic mobility[57], some authors have shown that when sialic acid is cleaved by purified neuraminidase, the electrophoretic mobility changes proportionally to sialic acid enzymatic release[8,48].

Because of the importance of the synaptic plasma membrane and because of the importance of

sialoglycoproteins and sialogangliosides at nerve cell surface coat, the present communication deals with the cytochemical identification of electrical charges at synaptosome surface by means of electron cytochemical and correlative biochemical techniques.

MATERIAL AND METHODS

Materials. Adult albino mice (local strain) were used throughout this work. The chemicals used for the biochemical determinations were purchased from Sigma Chemical Co. (St. Louis, Mo.)

The brains were obtained quickly after decapitation and pooled in a chilled homogenizer until enough tissue (usually 3-5 brains, cerebellum excluded) was collected for subcellular fractionation. A 10% (w/v) homogenate in 0.32M sucrose was prepared in a Teflon-glass homogenizer under gentle conditions (six strokes at approximately 850 r.p.m.)

Subcellular fractionation. The primary fractions (nuclei, mitochondria, microsomes and supernatant)

were obtained by following the procedure of De Robertis, Pellegrino de Iraldi, Rodríguez de Lores Arnaiz, and Salganicoff[17] and the mitochondrial fraction was subfractionated in a discontinuous sucrose density gradient, as described by Pérez de La Mora, Feria-Velasco, and Tapia[41]. After discarding the myelin fraction, the synaptosomal fraction was manually separated with a Pasteur pipette and diluted with about one-third of its volume of physiological saline solution and centrifuged at 100,000 g for 30 min. The pellet was resuspended in cold physiological saline solution for assays.

Incubation with colloidal iron. Ferric oxide solutions were prepared at pH 1.8 and 4.0 according to the method of Gasic, Berwick, and Sorrentino[19] in order to obtain positively (Fe(+)) and negatively (Fe(-)) charged colloidal iron solutions, respectively.

For incubation with colloidal iron, the synaptosome fraction was fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 at 4°C for

45 min[45]. After brief washing with the same phosphate buffer the synaptosomes were incubated in the colloidal iron solutions for 30 to 45 min at room temperature. The synaptosomes were rinsed twice in a 12% glacial acetic acid solution and subsequently in bidistilled and deionized water before postfixation. This was carried out in phosphate-buffered 1% osmium tetroxide for 1 hour at 4°C[38].

Some synaptosome samples were washed briefly in 2M sodium chloride solution before glutaraldehyde fixation and further incubation with the iron solutions, in order to avoid artifactual staining of material adsorbed to the synaptosome surface.

Methylation. The synaptosome fraction was fixed in buffered-2.5% glutaraldehyde[45] and briefly washed in 50% ethanol and absolute ethanol. Methylation was carried out by treating the samples with 0.1N HCl in absolute methanol for 24 hours at 60°C[30]. After rapid washing in 70% ethanol and bidistilled and

deionized water the samples were incubated with the Fe(+) solution and postfixed with osmium tetroxide.

Deamination. The synaptosome fraction was fixed in buffered-2.5% glutaraldehyde[45], washed twice with bidistilled and deionized water and incubated in a recently prepared solution of nitrose acid (1 gm $NaNO_2$, 30 ml of a 3% H_2SO_4 solution) for 48 hrs at 4°C in the dark[31]. After rapid washing in bidistilled and deionized water the samples were transferred to absolute ethanol and incubated at 60°C for 4 hours. These samples were washed in bidistilled and deionized water and incubated with the Fe(-) solution before postfixation with osmium tetroxide.

In some experiments the samples were incubated with purified Cl. perfringens neuraminidase (5 μg/mg synaptosome protein) for 60 min at 37°C (see below), washed twice in physiological saline solution and fixed with glutaraldehyde before the deamination process described above.

Neuraminidase treatment. Type V *Clostridium perfringens* neuraminidase (N-acetyl neuraminate glycohydrolase; EC 3.2.1.18) was purified according to the method described by Bernacki and Bosmann[2]. The enzyme activity was 0.7 units/mg (one unit= 1 μmole of sialyl lactose hydrolyzed/min at pH 5.0, at 37°C). The synaptosome preparation was briefly washed in physiological saline solution and incubated with neuraminidase (5 μg/mg synaptosome protein) at pH 7.0 in a shaking water bath at 37°C for 60 mins. After incubation the samples were washed in ice-cold 0.32 M sucrose and centrifuged at 7,000 g for 15 mins. The supernatant was used for measuring realased sialic acid[60] and the pellet was washed three to four times with physiological saline solution before fixation with buffered-glutaraldehyde and incubation with the Fe(+) solution. Postfixation was carried out with buffered-osmium tetroxide. Synaptosome samples incubated with boiled neuraminidase were used as controls.

Sialic acid in the synaptosomal fraction. Total sialic acid was determined in the synaptosomal fraction

following acid hydrolysis with 0.1N sulfuric acid at 80°C for 5 hours[22]. Sialic acid standards were treated similarly to correct for destruction of free sialic acid.

Electron Microscopy. After postfixation with osmium tetroxide the samples in all experiments were dehydrated in graded ethanols and embedded in Epon-812[34]. Thin sections in the silver color range of reflected light[40] were obtained in a Reichert OmU-2 or a Porter-Bloom MT-2 ultramicrotomes and collected in uncovered copper grids for observation without staining in a Philips EM-200 or a Zeiss EM-10 electron microscopes. Sections from the crude mitochondrial and synaptosomal fractions were stained with uranyl acetate[49] and lead citrate[56] for assessing their purity.

Analytical determinations. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall[33], whereas sialic acid was measured by the spectrophotometric method described by Warren[60].

R E S U L T S

The crude mitochondrial fraction consisted of myelin fragments, synaptosomes and mitochondria. Most elements in synaptosomal fraction were well preserved synaptosomes, disclosing numerous synaptic vesicles and intraterminal mitochondria.

Positive iron solution. When the samples were incubated with the Fe(+) solution, homogeneously distributed fine electrondense deposits, 5 to 20 nm diameter, were observed along the entire synaptosomal surface (Figs. 1 and 2). No particular differences in deposit distribution were noted between the synaptic contact area and the rest of synaptosomal membrane in the material examined (Fig. 3). When the synaptosomal fraction was washed with 2M NaCl solution before incubation with the Fe(+) solution, the synaptosomes appeared shrunken and fine electrondense deposits were homogeneously distributed at their surface (Fig. 4).

Methylation. Treatment of the synaptosomes with methanolic HCl solution before incubation with the Fe(+) solution prevented the appearance of surface deposits at the synaptosome membrane (Fig. 7).

Neuraminidase treatment. When the synaptosome preparation was incubated with purified Cl. perfringens neuraminidase, nearly 20% of total synaptosomal sialic acid was released (Table I). No electrondense deposits on synaptosome surface, including the synaptic contact area, were observed when the neuraminidase-treated synaptosomes were incubated with Fe(+) solution (Fig. 5).

Negligible amount of sialic acid was released when the synaptosomes were incubated with boiled purified neuraminidase (Table I), and electrondense deposits were observed at the surface of these synaptosomes after incubating them with the Fe(+) solution (Fig. 6).

Negative iron solution. The synaptosomes incubated with the Fe(-) solution after glutaraldehyde fixation,

disclosed a non-homogeneously distributed pattern of electrondense deposits at their surface (Fig. 8).

Deamination. No deposits were observed at synaptosome surface when deamination procedure was carried out before incubating the samples with the Fe(-) solution (Fig. 9). The same negative results were obtained when the incubation of the synaptosome preparation with the Fe(-) solution was performed after incubation with purified Cl. perfringens neuraminidase and deamination.

A summary of the ultrastructural findings in the present work appears in Table II.

D I S C U S S I O N

With high resolution cytochemical methods the surface coat has been demonstrated in nerve cells[23], specially at the synaptic elements[1,5,6,43] where some authors have shown the presence of specific structural and biochemical elements when compared with other nervous tissue components[5, 7, 25, 32, 53, 54]. Van

Nieu Amerongen, et al[53, 54] have reported the existance of a specific sialoglycoprotein (GP-350) isolated from the synaptosome membrane, which is absent in the membrane of other nervous tissue elements. This glycoprotein is identical in composition to that found in the soluble fraction of calf brain homogenates[55].

As to the topographic distribution of the carbohydrate residues in the nerve endings, Bittiger and Schnebli[3] employing ricin and concanavalin A labeled with ferritin showed the distribution of terminal groups supposedly containing galactose and others containing glucose or manose. The electrondense deposits were mainly concentrated at the intersynaptic region, particularly in the concanavalin A experiments. Whether this corresponds to the *in vivo* localization of the carbohydrate residues, or this distribution resulted from some molecule displacement at the synaptosome surface induced by the lectins, as it is the case of the capping phenomenon in other cells[26,42], is not known.

In the present work the distribution pattern of electrical charges at the synaptosome membrane is demonstrated by means of high resolution cytochemical techniques. The staining method with colloidal iron at various pH as a cytochemical staining for electron microscopy appears to be in part based upon electrostatic interactions of charged groups of tissue elements with colloidal iron[4,19,36]. Thus, when the colloid is negatively charged, labeling of the peripheral positively charged groups is observed. On the other hand, when positively charged colloidal iron is employed, the peripheral groups contributing to the net negative surface charge are stained. In our study, when the Fe(+) solution was used, a homogeneous distribution of electrondense deposits was observed at the synaptosome surface, corresponding to exposed electronegative charges. That the surface deposits were due to adsorption of extraneous charged material to the synaptosome surface could be ruled out by observing the electrondense deposits when the samples were washed with 2M NaCl

solution before incubation with the Fe(+) solution. Based upon the results obtained after methylation and after incubation with purified neuraminidase we can conclude that those electronegative charges most likely correspond to those conferred by the carboxyl group of sialic acid. This is further supported by the fact that in the sialidase experiments, sialic acid was demonstrated in the supernatant after centrifuging the enzyme-incubated samples. The enzyme-released sialic acid corresponded to nearly 20% of the total synaptosome sialic acid after severe acid hydrolysis. On the other hand, no sialic acid was released, while electrondense deposits were depicted at the synaptosome surface, when incubation with boiled neuraminidase was carried out. This is in keeping with what Bosmann and Carlson[8] have obtained after incubating the synaptosomes with purified neuraminidase (release of approximately 19% of the total synaptosome sialic acid). These authors observed that by using more than 4 μg neuraminidase per mg synaptosome protein there was no increase in the

release of sialic acid, and no modification in the electrophoretic mobility of synaptosomes (1.5 μm/sec/V/cm) was detected. This may indicate that some electronegative charges remained after neuraminidase treatment, probably conferred by sulfate or phosphate groups, mucopolysaccharides or even sialic acid residues not accessible by neuraminidase.

Apparently, there is more sialic acid residues per mg protein in the synaptic terminals than in the membranes of the rest of nervous tissue elements. This has been concluded from both, direct measurements[8,47,48] and indirect considerations. The latter are related to endogenous neuraminidase activity in the gray matter of cerebral and cerebellar cortex[20] and particularly associated with the synaptosome membrane in adult bovine gray matter[46]. It has been postulated that neuraminidase activity, is high where there is a high metabolism of sialic acid[11]. Both, gangliosidic and protein-bound sialic acid have been considered important elements very likely involved in transmission processes[29,51].

When the Fe(-) solution was used, an irregular and non-homogeneous distribution pattern of dense deposits were seen at the synaptosome surface, which disappeared when deamination was performed before staining with the Fe(-) solution. This suggests that those surface positive charges are probably conferred by exposed amino groups. Whether the staining pattern with the Fe(-) solution according to the methods of Gasic, et al[19] reflects the scarcity of positively charged groups at the synaptosome surface coat, cannot be positively asserted from the present work. However, other authors have observed a similar irregular distribution pattern of positive surface charges in other cells incubated with the Fe(-) solution[9]. An alternative possibility may be that free carboxyl and other negatively charged groups, determining the net negative charge of the synaptosome surface, prevent the staining of the basic groups by predominant electrostatic repulsion of the negatively charged iron particles. However, neuraminidase

treatment before deamination followed by incubation with the Fe(-) solution did not change the labeling pattern of the synaptosome surface in the present work.

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TABLE I.

SIALIC ACID IN MOUSE BRAIN SYNAPTOSOMES AND SIALIC ACID RELEASED BY TREATMENT WITH PURIFIED *Cl. perfringens* NEURAMINIDASE

Data represent means ± SEM for the number of experiments shown in parentheses.

	After severe acid hydrolysis	Release by treatment with purified neuraminidase (5 μg/mg*)	
			Boiled
nmoles/mg synaptosome protein	4.3 ± 0.008 (6)	0.924 ± 0.075 (6)	0.005 ± 0.002 (6)

* synaptosome protein

TABLE II.

ULTRASTRUCTURAL FINDINGS WHEN SYNAPTOSOMES WERE TREATED WITH COLLOIDAL IRON SOLUTIONS UNDER VARIOUS CONDITIONS.

Fe(+) = Colloidal iron solution, pH 1.8

Fe(-) = Colloidal iron solution, pH 4.0

Procedure	Electrondense deposits at synaptosome surface
Fe(+)	Homogeneous
Methylation-Fe(+)	None
Neuraminidase-Fe(+)	None
Boiled neuraminidase-Fe(+)	Homogeneous
Fe(-)	Non-homogeneous
Deamination-Fe(-)	None
Neuraminidase-Deamination-Fe(-)	None

LEGENDS OF FIGURES

FIGURE 1. Homogeneous labeling pattern was observed at synaptosome surface after incubation of synaptosomal fraction with the Fe(+) solution. Unstained section (18,000x).

FIGURE 2. Fine electrondense deposits (arrow) were homogeneously distributed at synaptosome surface after incubation with the Fe(+) solution. Unstained section (40,000x).

FIGURE 3. No particular distribution pattern of deposits was observed at the synaptic contact area (arrow) after incubation with the Fe(+) solution. S= synaptic terminal containing a mitochondrion (m) and clear synaptic vesicles (v). Unstained section (40,000x).

FIGURE 4. Synaptosome fraction briefly washed in 2M NaCl before fixation and incubation with the Fe(+) solution. Although the synaptosomes (S) appear shrunken, fine electrondense deposits (arrows) are disclosed homogeneously distributed at their surface. m = intraterminal mitochondria; v = synaptic vesicles. Unstained section (40,000x).

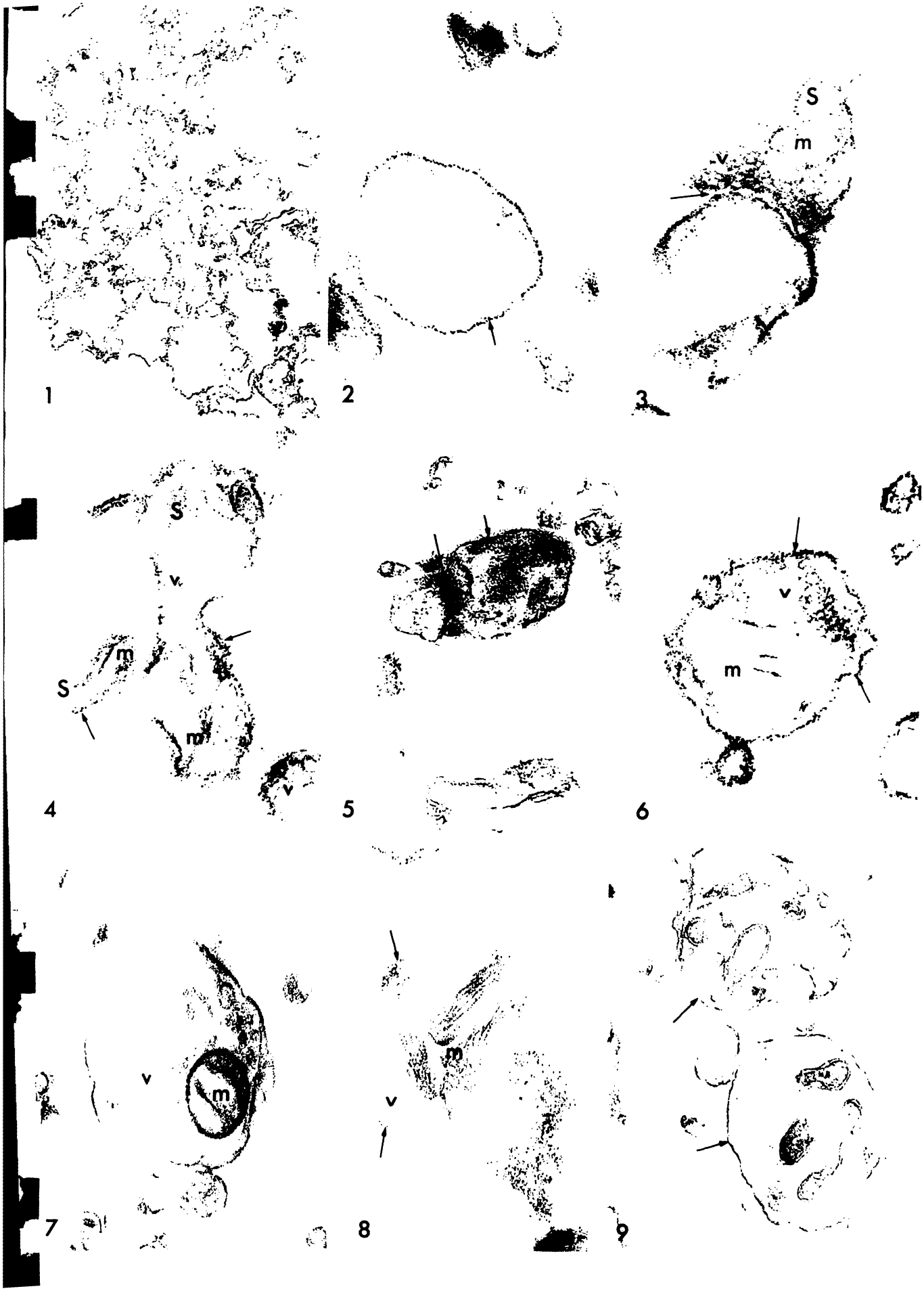
FIGURE 5. No deposits were observed at synaptosome surface (short arrow) when the samples were incubated with purified *Cl. perfringens* neuraminidase before fixation and incubation with the Fe(+) solution. Long arrow = synaptic contact area. Unstained section (30,000x).

FIGURE 6. Electrondense deposits (arrows) appear homogeneously distributed at synaptosome surface after incubation with boiled purified *Cl. perfringens* neuraminidase prior to fixation and incubation with the Fe(+) solution. m = intraterminal mitochondria; v = synaptic vesicles. Unstained section (40,000x).

FIGURE 7. No deposits were observed at the synaptosome surface when the samples were treated with methanolic HCl solution (methylation procedure) before incubation with the Fe(+) solution. m = intraterminal mitochondria; v = synaptic vesicles. Unstained section (40,000x).

FIGURE 8. Non-homogeneously distributed pattern of electrondense deposits (arrows) was observed at synaptosome surface when the synaptosomes were incubated with the Fe(-) solution. m = intraterminal mitochondria; v = synaptic vesicles. Unstained section (40,000x).

FIGURE 9. No electrondense deposits were observed at synaptosome surface (arrows) when the synaptosomes were subjected to a deamination procedure before incubation with the Fe(-) solution. Unstained section (40,000x).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