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ABSTRACT

Synaptic junctional fractions were separeted from rabbit brain by procedures based on composite of the methods of Cotman and Taylor /1972/, Orosz et al. /1973, 1974/ and Lisman et al. /1979/. Thin layers of cerebral cortexes were homogenized to prepare a crude mitochondrial-synaptosomal fraction. The sedimentation rate of mitochondria and mitochondria-containing synaptosomes was increased so as to raise the density of mitochondria by depositing an insoluble dense formazan inside the mitochondria after iodonitrotetrazolium treatment. The synaptical plasma membrane fraction isolated by this method contained no mitochondrial contamination. After Trion X-100 treatment the detergent insoluble residues were centrifuged through discontinuous sucrose gradients. A great enrichment of morphologically identifiable intact synaptic junctions was observed in some of the obtained interface layers.

АННОТАЦИЯ

С помощью процедуры, разработанной по методам Котмана, Тейлора /1972/, Ороса и др. /1973,1974/, а также Лисмана и др. /1979/, из мозга кроликов была сепарирована синаптическая юнкциональная комплексная фракция. Тонкие слои, тщательно отделенные от белого вещества коры большого полушария, были гомогенизированы, и из них получена сырая фракция митохондрия /синаптосомы/. В митохондриях и в синаптосомах, содержащих митохондрий, формазановые связи, созданные с помощью обработки йодонитротетразолием, значительно увеличили седиментацию этих частиц. Сепарированная этим методом синаптическая плазма-мембрановая фракция не содержала митохондриального загрязнения. После обработки тритоном-X-100 мембрановая суспензия, не растворимая в детергенте, была дальше очищена с применением ступенчатого сахарозного градиентного центрифугирования. В отдельных слоях на границах раздела фаз были получены морфологически идентифицируемые чистые фракции интактных синаптических юнкциональных комплексов.

KIVONAT

Szinaptikus junkcionális komplex frakciót szeparáltak nyulak agyából Cotman és Taylor /1972/, Orosz és mtársai /1973, 1974/ és Lisman és mtársai /1979/ módszerei alapján kidolgozott eljárás segitségével. A fehérállománytól gondosan elválasztott nagyagyi kéregállomány vékony rétegeit homogenizálták,és ebből nyers mitokondrium /-szinaptoszóma/ frakciót állitottak elő. A mitokondriumokban és a mitokondriumot tartalmazó szinaptoszómákban jodonitrotetrazólium kezeléssel létrehozott formazán kötések jelentősen megnövelték e részecskék szedimentációját. Ezzel a módszerrel szeparált szinaptikus plazma-membrán frakció nem tartalmazott mitokondriális szennyeződést. Triton X-100 kezelés után a detergensben oldhatatlan membrán szuszpenziót lépcsős szaharóz gradiens centrifugálással tovább tisztitották. A fázishatárokon kapott egyes rétegekben morfológiailag identifikálható, intakt szinaptikus junkcionális komplexek tiszta frakcióit nyerték.

Introduction

The synaptic junctional membrane region of the nerve cells as the site of the inter-neuronal connections is of particular interest among the membranes of the brain. Biophysical studies of this region may lead to a better understanding of the basic nervous communication processes. However, investigation of events involved in these processes requires subcellular membrane fractions pure enough for biophysical analyses to be performed with confidence so that what is measured reflects the properties of the genuine junctional components and not those of contaminants.

Another necessity is to take the data from such broken cell preparations which were treated with various chemicals and somehow translate them back to the operation of synaptic membranes in vivo. It seems that one of the best ways out of this kind of problem is to use antisera. A powerful type of analysis using antisera for immuno electron microscopic technique needs to be used to corroborate marker enzyme studies and to enable cross--checking with one another.

Bearing in mind all the above mentioned difficulties the procedure we followed is a combination of the methods of Cotman and Taylor /4/, Orosz et al. /14,15/, and Lisman et al. /11/.

Cotman and Taylor 1972 /4/ and Davis and Bloom 1973 /5/ used a new approach to eliminate mitochondrial contamination. The clue to success using this method is to raise the density of mitochondria to a high degree after iodonitrotetrazolium salt treatment. Orosz et al. 1973,1974 /14,15/ examined the specificity of the same synaptic

junctional fractions with different immunological techniques, and their electron microscopic version for determining whether or not a membrane-antigen is associated with synaptic junctional structures was particularly useful. A detailed viability study was made by Lisman et al. 1979 /11/ who found that after Triton X-100 detergent and iodo-nitrotetrazolium treatment of the fractions: the marker enzyme activity of nine important markers remained unchanged in the isolated synaptic membranes. Recently, Salvaterra and Mathews 1980 /16/ characterized these membrane fractions with respect to the relative enrichment of neurotransmitter receptors determined by different radio--ligand binding assays.

Materials and methods

Adult rabbits were decapitated: thin layers of cerebral cortexes were macroscopically excised and placed in ice cold 0.32 M sucrose containing 1 mM $MgCl_2$ pH 7.0. Subsequent homogenization was performed by an Elvehjem-Potter type homogenizer/Braun-Melsungen/ to give a 30% /w/v/ suspension. /The procedure is illustrated in Scheme 1./. The pestle rotated at 600 rpm for 14 strokes up and down. The homogenate was diluted to 10% /w/v/ and centrifuged using a Beckman JA 14-rotor at 1000 g for 5 min to remove nuclear and other greater cellular debris. Pellets were resuspended and washed, then discarded. The supernatants were shaved and centrifuged at 17000 g for 5 min to prepare a crude mitochondrial fraction. The mitochondrial pellet was homogenized by a Potter homogenizer rotated at 200 rpm, the suspension was made by adding distilled water and 50 µM Ca-acetate and adjusted to pH 7.5. The hypotonic shock was at 4 ^{o}C for 20 min. After hypotonic treatment, the suspension was centrifuged at 17000 g and the pellet was resuspended in iodonitro-tetrazoliumchloride /INT/: INT 1 mM, 40 mM Tris-HCl pH 7.5 60 mM sodium succinate, 50 µM Ca-acetate in

10 vol/g initial weight. The INT-suspension was kept 30°C for 20 min.

The lysed and INT treated crude mitochondrial fraction was pelleted at 10000 g, washed with 0.1 M sucrose to reduce the salt concentration. After that, it was centrifuged in a Beckman JA 20 rotor at 36000 g 15 min. The pellet was resuspended in 0.32 M sucrose /50 µM Ca-acetate pH 7.0/ and applied to a discontinuous sucrose gradient of 0.8 M, 0.9, 1.0 and 1.2 M each containing 50 µM Ca-acetate. The gradients were spun at 70000 g for 1h 45 min using MSE Prepspin 65 ultracentrifuge SW 70 rotor. The fractions /Fig.1/ were designated by an MSE gradient extraction unit. The SPM-fraction was diluted with 50 µM Ca-acetate banded at 1.0-1.2 M and pelleted in an MSE SW 6.5 rotor at 60000 g for 25 min. The SPM-pellet was resuspended in 2 mM Bicine buffer of pH 7.5. This enriched synaptic plasma membrane fraction was treated in Bicine buffer containing 24 mM Bicine pH 7.5 and 2 mM EDTA with Triton X-100 at 4°C for 20 min. The Triton-protein ratio was 2:1 Triton mg/protein mg. The membrane solution was layered on a sucrose density gradient: 1.0, 1.2, 1.4 M and 1.5 M; each solution contained 50 µM Ca-acetate. The gradients were spun at 65000 g in an MSE SW 6.5 rotor for 1hr 15 min to isolate synaptic junctional complexes /isolation procedure is illustrated in Scheme 2/. The bands at 1.2-1.4-1.5 interfaces were collected and diluted with Tris-HCl buffer pH 7.4-7.5. Protein determination was carried out by Lowry's method /12/.

Samples for electron microscopy were prepared by washing with a buffer and centrifuged at 100000 g. The samples were fixed with 1% glutaraldehyde and para-formaldehyde for 2h at 4°C and postfixed with 1% osmium tetroxyde /1h 4°C/. They were then dehydrated in a graded series of ethanol solutions and embedded in Durcupan ACM. Sections were stained with uranyl acetate and lead citrate.

Examinations were carried out by a JEOL-100B electron microscope.

Results and discussion

Two general approaches have been used in efforts to isolate subfractions of nervous membranes enriched in synaptic domains. In one case when nervous system tissue is homogenized the synaptic boutons are sheared from their axons and reseal retaining their contents /6,8/. These synaptosomes were obtained by density gradient centrifugation of the crude mitochondrial fractions on sucrose /13,20/, Ficoll-sucrose /1,7,3,14/, caesium chloride /10/, sodium ditriozate /17/, silica gel /9/ gradients and by electrophoresis /18/. The synaptosomes are then lysed by osmotic shock in hypotonic solutions and the synaptic membrane enriched fractions isolated by equilibrium density gradient centrifugation.

In the alternative strategy, a crude mitochondrial fraction containing synaptosomes is isolated, lysed by osmotic shock and treated by iodonitrotetrazolium salt. After multiple washing steps this fraction was subfractionated by sucrose density gradient centrifugation. Here, we used the latter procedure /Scheme 1/ because it seems to have more morphologically identifiable synaptic junctions. In the course of our experiments the so called synaptic plasma membrane fraction /SPM/ which banded after centrifugation at the 1.0 M - 1.2 M sucrose interface was collected. Figure 2 is an electron micrograph of this fraction where numerous synaptosomes, dumbbell-shaped structures of the postsynaptic membranes, some postsynaptic densities, and also some lipofuscine granules are visible. No mitochondria could be detected in this fraction.

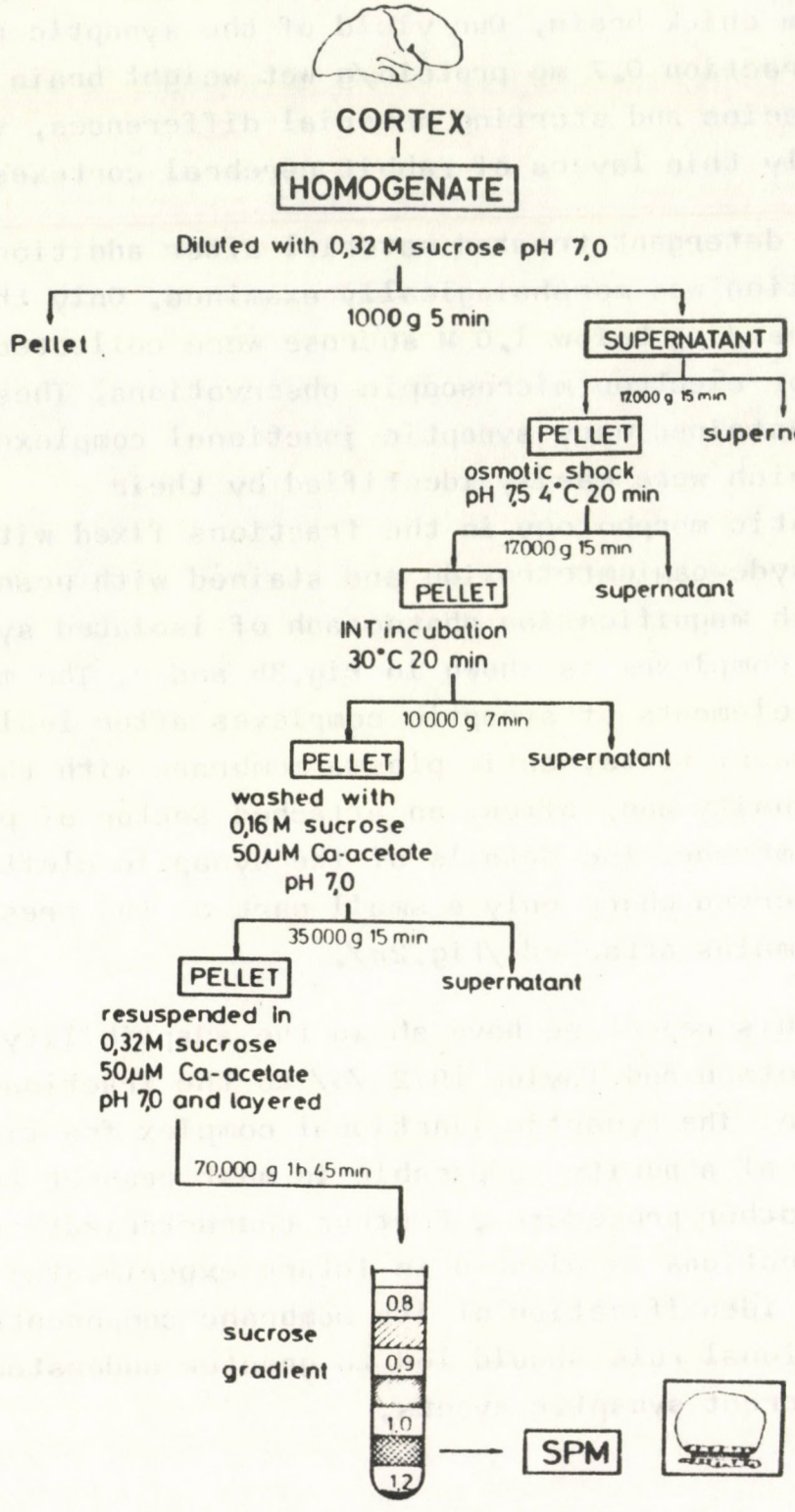
Using rat brain, Cotman and Taylor 1972 /4/ obtained 3 mg protein/g wet weight forebrain of material banding at the 1.0-1.2 M sucrose interface, whereas Davis and Bloom

1973 /5/ obtained only about 2 mg protein/g brain wet weight, and Babitch et al. 1976 /2/ obtained 0.9 mg protein/g wet weight from chick brain. Our yield of the synaptic plasma membrane fraction 0.7 mg protein/g wet weight brain may reflect species and starting material differences, viz, we used only thin layers of rabbit cerebral cortexes.

The detergent treated material after additional centrifugation was morphologically examined. Only those fractions banding below 1.0 M sucrose were collected and prepared for electron microscopic observations. These fractions contained many synaptic junctional complexes /Fig.3a/ which were easily identified by their characteristic morphology in the fractions fixed with glutaraldehyde-osmiumtetroxide and stained with uranyl lead ions. A high magnification photograph of isolated synaptic junctional complexes is shown in Fig.3b and c. The major structural elements of synaptic complexes after isolation are a prominent postsynaptic plasma membrane with the post-synaptic density and, often, an attached sector of pre-synaptic membrane. The details of the synaptic cleft can best be observed where only a small part of the presynaptic membrane remains attached /Fig.2c/.

In this report we have shown the adaptibility of the method of Cotman and Taylor 1972 /4/ to the fractionation of rabbit brain. The synaptic junctional complex fraction obtained is of a purity comparable in most respect to that derived by other procedures. Further characterization of the synaptic junctions is planned in future experiments: biophysical identification of its membrane components and their functional role should led to greater understanding of the different synaptic events.

ISOLATION OF SYNAPTIC PLASMA MEMBRANES



Scheme I.

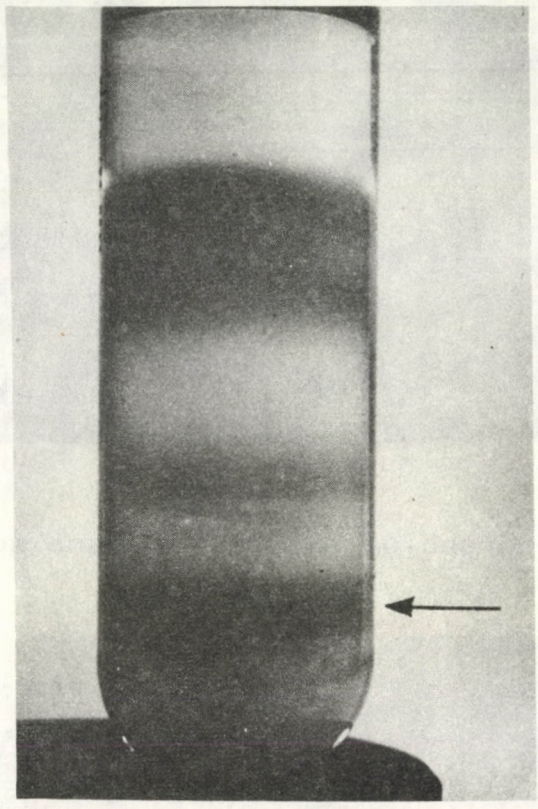


Figure 1.

Discontinuous sucrose density gradient after ultracentrifugation. SPM enriched fraction banded at the 1.0-1.2 M sucrose interface.

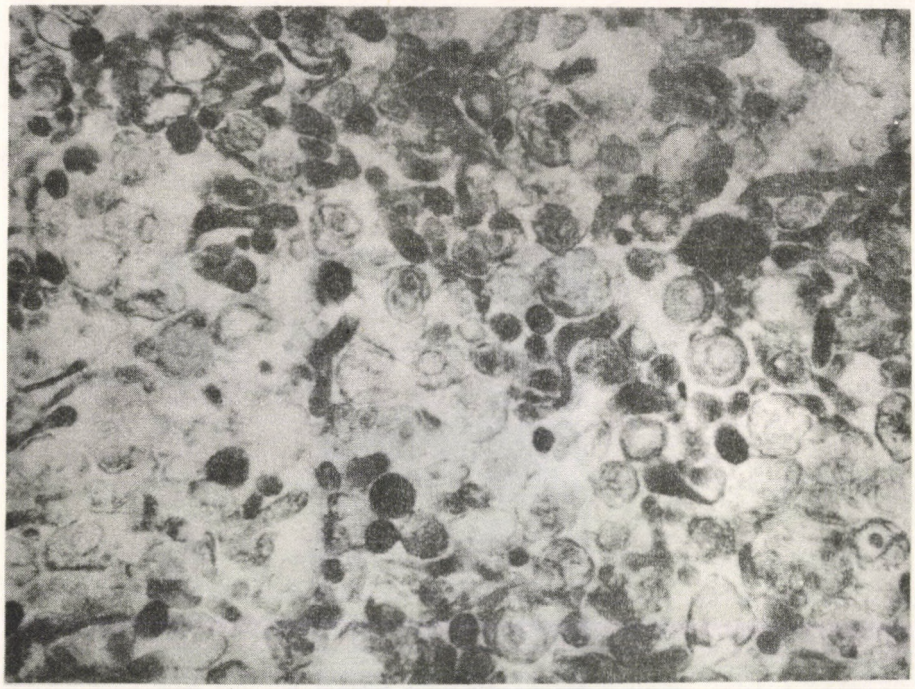


Figure 2a.

Electron micrograph of synaptic plasma membrane fraction. X 38 000.

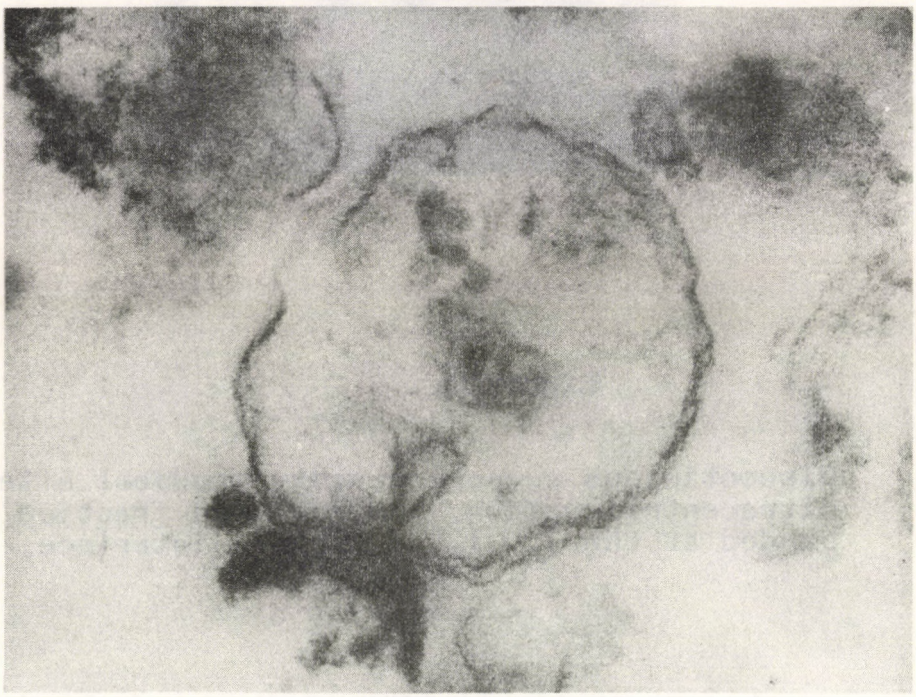
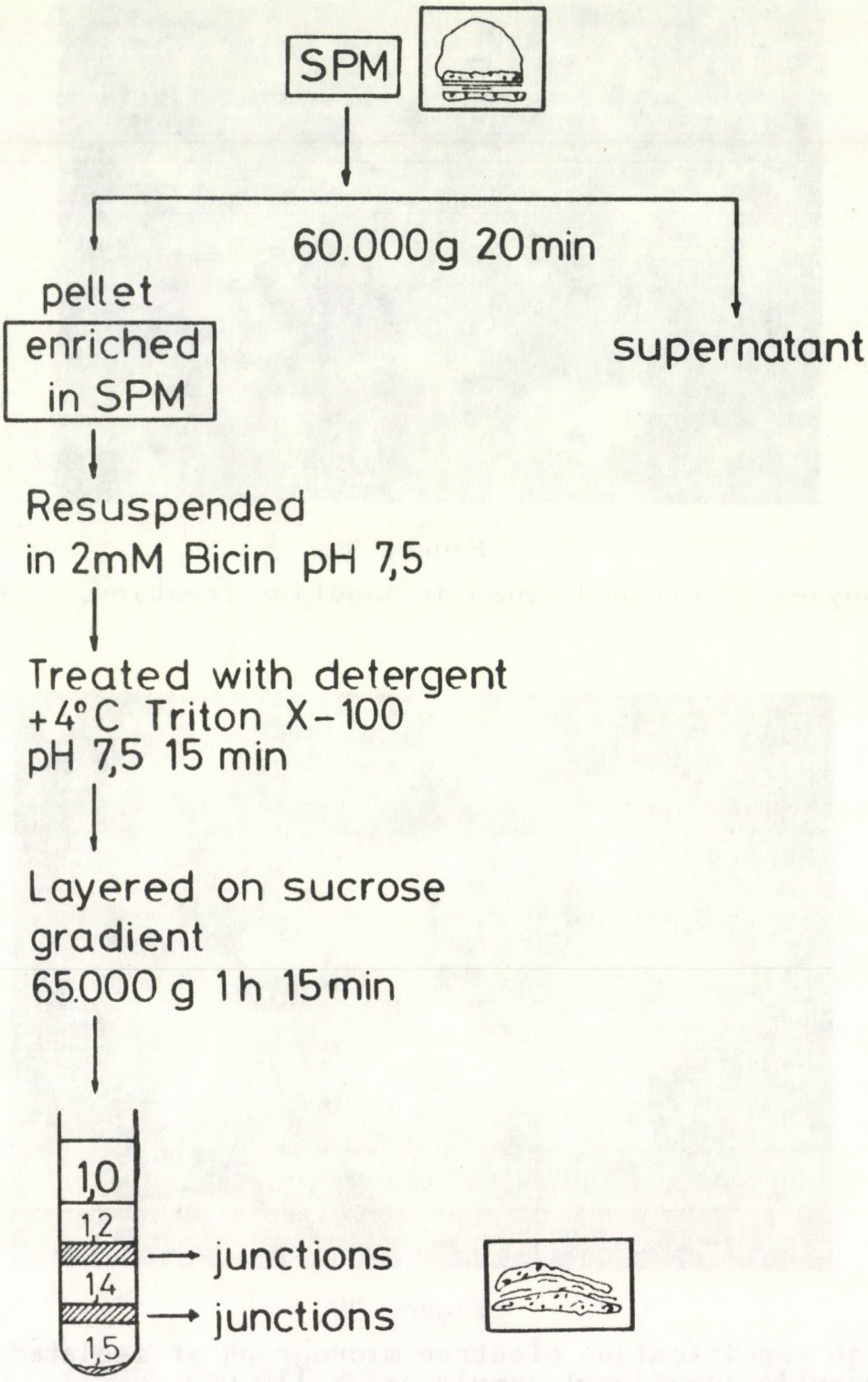


Figure 2b.

High magnification photomicrograph of an isolated synaptic membrane. X 85 000.

ISOLATION OF SYNAPTIC JUNCTIONS



Scheme II.

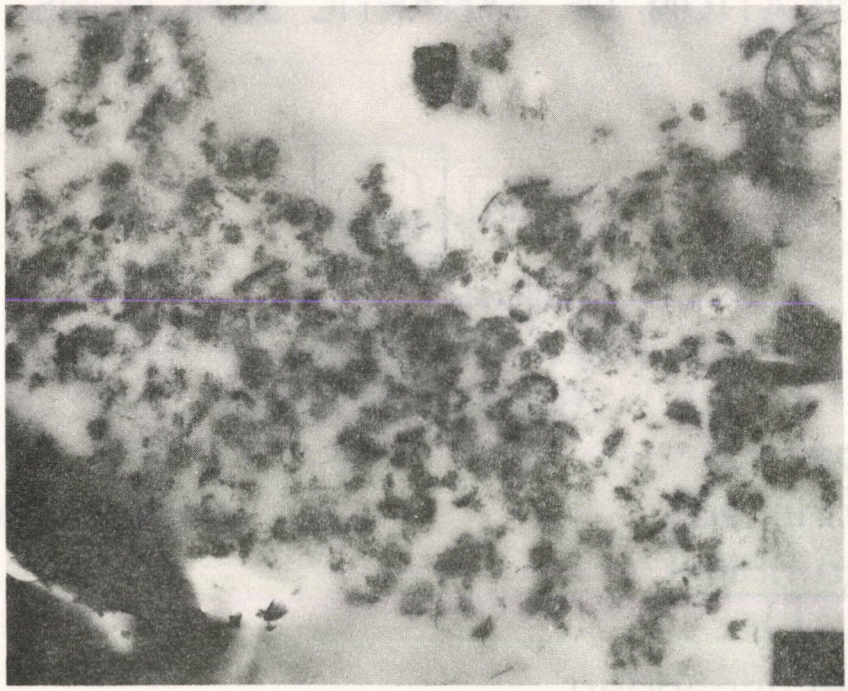


Figure 3a.
Uranyl-lead stained synaptic junction fraction. X 20 000.

Figure 3b.
High magnification electron micrograph of isolated synaptic junctional complexes. X 110 000.

Figure 3c.

High magnification electron micrograph of one isolated synaptic junctional complex. X 200 000.

References

1 Abdel-Latif, A.;, /1966/. A simple method for isolation of nerve-ending particles from rat brain. Biochim.Biophys.Acta 121,403-406.

2 Babitch,J.A., Breithaupt, T.B., Tien-Cheng Chiu, Geradi,R. and Helseth, D.L. /1976/. Preparation of chick brain synaptosomes and synaptosomal membranes. Biochim.Biophys.Acta 433,75-89.

3 Cotman, C.W. and Matthews, D.A. /1971/. Synaptic plasma membranes from rat brain synaptosomes: Isolation and partial characterization. Biochim.Biophys.Acta 249,380-394.

4 Cotman, C.W. and Taylor, D. /1972/. Isolation and structural studies on synaptic complexes from rat brain. J. Cell Biol. 55,696-711.

5 Davis, G.A., and Bloom, F.E. /1973/. Isolation of synaptic junctional complexes from rat brain. Brain Res. 62,135-153.

6 De Robertis, E., A.P.De Iraldi, G.R.De Lores Arnaiz and L.Salganicoff. /1962/. Cholinergic and non-cholinergic nerve endings in rat brain. J. Neurochem. 9,23-35.

7 Fahn, S., Rodman, J.S., Cote, L.J. /1969/. Association of thyrosine hydroxylase with synaptic vesicles in bovine caudate nucleus. J. Neurochem. 16,1293-1300.

8 Gray E.G. and V.P. Whittaker /1962/. The isolation of nerve endings from brain and electron microscopic study of cell fragments derived by homogenization and centrifugation. J.Anat. 96,79-88.

9 Lagercrantz, H., and Pertoft, H. /1972/. Separation of catecholamine storing synaptosomes in colloidal silica density gradients. J. Neurochem. 19,811-823.

10 Levitan, I.B., Mushynski, W.E. and Ramirez, G./1972/. Highly purified synaptosomal membranes from rat brain. Preparation and characterization. J.Biol.Chem. 247, 5376-5381.

11 Lisman, J.J.W., J.De Haan and B.Overdijk /1979/.
Isolation of Lysosomes from Brain Tissue.
Biochem. J. 178,79-87.

12 Lowry, O.H., Rosenbrough N.J., Fan, A.L.,
Tandall, R.J. /1951/.
Protein measurement with the Folin phenol reagent.
J.Biol.Chem. 193, 265.

13 Mc Bride, W.J., Mahler, H.R., Moore, W.J., and
White, F.P. /1970/.
Isolation and characterization of membranes from
rat cerebral cortex.
J.Neurobiol. 2,73-92.

14 Orosz A., J. Hámori, A.Falus, E. Madarász, I. Lakos
and G. Ádám /1973/.
Specific antibody fragments against the postsynaptic
web.
Nature, Neur.Biol. 245,18-19.

15 Orosz A., E. Madarász, A.Falus and G. Ádám /1974/.
Demonstration of detergent soluble antigen specific
for the synaptosomal membrane fraction isolated from
the cat cerebral cortex.
Brain Res. 76,119-131.

16 Salvaterra P.M., D.A. Matthews /1980/.
Isolation of rat brain subcellular fraction enriched
in putative neuro transmitter receptors and synaptic
junctions.
Neurochem.Res. 5,181-195.

17 Tamir, H., Rapport, M.M. and Roizin, L. /1974/.
Preparation of synaptosomes and vesicles with
sodium ditriozate.
J.Neurochem. 23,943-949.

18 Tkachenko, A.N. /1972/.
Isolation of synaptosomal membrane from animal brains.
Biokhimiya 37,248-254.

19 Whittaker, V.P., Michaelson, I.A. and Kirkland,R.J.A.
/1964/. The separation of synaptic vesicles from nerve
ending particles /'synaptosomes'/.
Biochem.J. 90,293-303.

20 Whittaker, V.P. /1966/.
Some properties synaptic membranes isolated from the
central nervous system.
Ann.N.Y.Acad.Sci. 137,982-998.

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