Synthesis of Membrane Protein in Slices of Rat Cerebral Cortex

SOURCE OF PROTEINS OF THE SYNAPTIC PLASMA MEMBRANES*

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SUMMARY

Protein synthesis was studied in slices from rat cerebral cortex and localized in various purified subcellular membrane fractions isolated after incubation with L-leucine. A synaptic mitochondrial fraction isolated from a several times washed crude mitochondrial pellet showed very little contamination by free membranes, and the synaptic membrane fraction isolated from it was estimated to contain about 50% of this component. Leucine incorporation into all fractions was highly sensitive (> 95%) to emetine except for the cell and synaptic mitochondrial subfractions. They were only 60 to 70% inhibited by emetine and showed 10 to 20% inhibition by chloramphenicol which probably was due to those proteins synthesized in situ by the mitochondria. The net rate of incorporation of labeled protein into the synaptosomal and synaptic membrane subfractions was low, and axonal flow was excluded as a significant source of this label. On the basis of autoradiography it was concluded that the majority of the protein label was contributed by free and membrane-enclosed ribosome-containing contaminants in these fractions.

Unlabeled and labeled subcellular fractions were also analyzed by electrophoresis in the presence of sodium dodecyl sulfate. The staining profiles of the microsomal and synaptic membrane subfractions were nearly identical, whereas those of synaptic mitochondria and the soluble proteins of the cell bodies were unique. The labeling of all proteins was blocked by emetine, except for the synaptic mitochondria (cell mitochondria were not examined). These products of mitochondrial protein synthesis exhibited three labeling peaks, the major one with a molecular weight of approximately 38,000. In the absence of emetine, slices incorporated amino acids into soluble and microsomal proteins with high specific activity. The labeled proteins of the soluble fraction were more highly concentrated in the range of molecular weights smaller than 50,000 than were those in the microsomal fraction, and it was concluded that a considerable portion of the protein-synthesizing machinery of the brain is geared to form soluble proteins. The gel patterns of labeled microsomal and synaptic membranes were consistent with contamination by the former contributing most of the protein label in the latter. In contrast to many of the opinions expressed in the current literature it was concluded that presynaptic nerve endings do not synthesize protein by any mechanism other than one due to their mitochondria.

Neuronal cell bodies constitute a very small proportion of the total cellular volume (5%) of the fully developed cerebral cortex. The majority is contributed by the neuropil, a feltwork of intermingled and interconnected processes of neurons, where most of the synaptic junctions are located. The spatial separation of neurons from their processes and the absence of ribosomes from axons, presynaptic nerve endings, and the terminal branches of dendrites require that proteins be supplied to the neuropil, for brain proteins are known to exhibit turnover. The overwhelming majority of this protein supply is provided by neuronal cell bodies, where free and membrane-bound polysomes are abundant, and is then transported to the peripheral processes. These proteins are transported down the interior of axons (and dendrites) at at least two rates—a slow rate of a few millimeters per day and a fast rate of several hundred millimeters per day. In addition there is also some less substantive evidence for a small amount of in situ protein synthesis in axons and presynaptic nerve endings.

The question of the true extent and nature of such local synthesis is an important one, for it may provide a molecular basis for synaptic modification, events thought to involve changes at both the morphological and biochemical level. It has also proved to be highly vexing and controversial. One possible approach to a resolution of this problem has been the use of tissue slices of rat cerebral cortex for studying the kinetics of incorporation of a radioactive precursor into the...
membrane proteins of defined subcellular compartments, especially those presumed to be of synaptic origin (19). Brain slices have been used in many metabolic studies, including ones on the biogenesis of macromolecules, and these investigations have shown that these preparations retain many attributes of the living tissue (20–22). Particularly pertinent are observations based on electron microscopy that demonstrate minimal neuronal damage after incubation. The extent of release of protein from the cut surfaces into the incubation medium is small, and it is further reduced by the high proportion of narrow axons and dendrites capable of rescaling; it has been estimated (21) that the fraction of surfaces disturbed (cut and rescaled) accounts for less than 1% of the total surface area in the slices.

By adopting sufficiently discriminating techniques of subcellular fractionation to homogenates prepared from such slices after incubation, it should be possible to isolate and characterize the protein metabolism of isolated nerve endings (synaptosomes) and their constituents. However, it is also apparent that any results obtained might be difficult, if not impossible, to interpret due to the extraordinary heterogeneity of the starting homogenate, particularly with respect to its content of materials from different cell types of both neuronal and glial origin. This difficulty is obviated by several recent studies, which have shown that, at least in the adult rodent brain, neurons exhibit a much greater capacity for protein synthesis than do glia. This conclusion is based on experiments using radioautography in vivo (23) and on comparisons of the synthetic activity of isolated neurons and glia from fresh brain (24) and of neuronal and glial fraction isolated after incorporation studies (25, 26) with brain slices similar to those described below.

In this report we examine the problem by presenting data on the incorporation of radioactive leucine into synaptosomes, their membranes, and other membranous organelles; all isolated by techniques designed to minimize cross-contamination, and the effect thereon of site-specific inhibitors of protein synthesis and axonal transport. We attempt to localize the site of synthesis on specific organelles directly by means of radioautography and, finally, endeavor to determine the nature of the products by means of electrophoresis of subunits in acrylamide–sodium dodecyl sulfate gels.

**EXPERIMENTAL PROCEDURES**

**Preparation and Incubation of Tissue Slices**—These were as described previously (19), except that we modified the medium as follows in order to maintain a constant pH throughout the incubation at 7.2 (all concentrations are mm): NaCl, 50; KCl, 5.1; CaCl₂, 0.75; MgSO₄, 1.3; glucose, 10; sodium phosphate buffer, 50. Slices from six cortices were used per experiment, arranged pairwise for removal at different times or for comparing different parameters. Slices from individual cortices were incubated separately and pooled at the end of the incubation.

**Subcellular Fractionation of Slices**—All solutions were prepared with glass-distilled water containing 10 mm l-leucine, pH 7.4. The leucine was added to reduce any nonspecific adsorption of l-[H]leucine to the subcellular particles, and it also served as a buffer. In preliminary experiments we observed that when calcium, magnesium, or phosphate salts or Tris buffer solutions (1 to 5 mM) were added to the fractionation solutions, extensive clumping of the brain fractions occurred, whereas leucine prevented this effect. The pH of all final solutions was adjusted to 7.4 on the day of the experiments, and all operations were at 4°C.

The duplicate preparations were combined by resuspension in 20 ml of 0.32 m sucrose (10 mm leucine, pH 7.4), and the slices were then pelleted in the 30 rotor at 1,000 × g for 7 min using a Beckman model L, L-2, or L-65-B centrifuge for this and all other centrifugation steps. The samples were next homogenized in 10 ml of 0.32 m sucrose with a glass and Teflon homogenizer (Arthur Thomas Co.) with a clearance of 0.15 mm. Ten pluses at 200 rpm were used; another 10 ml of 0.32 m sucrose were then added and the suspension was mixed with 5 additional pluses. The P₁ fraction was obtained by centrifuging the homogenate in the 30 rotor at 1,000 × g (3,000 rpm) for 7 min. This pellet consisted of red blood cells, nuclei, and cell debris. The supernatant (S₁) was then spun again, and care taken to eliminate the white layer on top of the P₁ pellet from entering the S₁ suspension. In all experiments S₁ was further purified by a second centrifugation at 1,000 × g and the pellet discarded to ensure that most of the nuclei and cell debris had been removed. The S₂ fraction was then centrifuged in the 30 rotor at 15,000 × g (12,000 rpm) for 20 min to obtain the P₂ pellet. This was the crude mitochondrial-synaptosomal fraction, but this fraction also contained other particles such as myelin and microsomes. P₃ was then washed by pouring off the supernatant (S₃) and resuspending the pellet in 15 ml of 0.32 m sucrose with a Vertex-Genie (Scientific Industries, Inc.). The suspension was then centrifuged at 13,000 × g for 20 min to obtain the washed P₃ pellet, which was then subjected to three additional washes by the same procedure. The P₄ pellet was isolated from S₄ by centrifugation at 79,488 × g (30,000 rpm) for 90 min after a preliminary centrifugation at 13,000 × g for 20 min to pellet out large particle contaminants. The second pellet (P₅) represented the microsomal fraction, consisting of small membrane fragments derived mainly from the intracellular membranes (rough and smooth endoplasmic reticulum, Golgi apparatus, etc.) and ribosomes. The final supernatant (S₅) constituted the soluble fraction. The various samples were stored by freezing at −20°C, the particulate ones after resuspension in 0.32 m sucrose.

**Determination of Radioactivity**—The incorporation of [H]leucine into protein was measured in duplicate by the procedure of Mann and Novelli (27). The radioactivity soluble in trichloroacetic acid was determined by subtracting the radioactivity precipitable by this agent in the homogenate from the total radioactivity in the homogenate. A zero time control experiment showed no significant labeling in the precipitable protein. The samples were counted on a Mark I Nuclear-Chicago scintillation counter with an efficiency of about 30%.

For polyacrylamide gel electrophoresis the gel slices were counted as described by Tashler and Epstein (28). The slices were dissolved in 0.5 ml of 30% hydrogen peroxide at 50°C for 6 to 12 hours and a mixture of Triton X-100 and toluene (1:2) was used for counting. Quench corrections were made by the external standard method; however, this was probably not necessary as the efficiencies in different fractions showed little variation. The extent of recovery of the total label applied to gels was greater than 94%, and its recovery from duplicate gels was virtually identical (±5%).

**Determination of Protein and Enzymatic Activities**—Protein was determined by the method of Lowry et al. (29) using bovine serum albumin as a standard. The ouabain-sensitive (Na⁺ + K⁺)-activated ATPase was assayed according to Verity (30). NADPH:cytochrome c reductase and rotenone-insensitive NADH:cytochrome c reductase were measured as described by Sottocasa et al. (31). Rotenone was added to a final concentration of 10 μg per ml. Cytochrome oxidase was determined by the method of Duncan and Mackler (32), acid phosphatase was assayed according to Cotman and Matthews (33) using p-nitrophenyl phosphate as the substrate, monamine oxidase was assayed according to Earl and Korner (34), and lactate dehydrogenase according to Johnson (35). RNA was determined by the method of Fleck and Begg (36) using soluble yeast RNA as a standard.

**Polyacrylamide Gel Electrophoresis**—Electrophoresis was performed after the procedure of Weber and Osborn (37). The gel concentration was 7.5%; acrylamide (N,N′-methylenebisacrylamide); acrylamide = 2.5%), and N,N′,N″,N‴-tetramethylbiguanide and ammonium persulfate were used to polymerize the gels in a phosphate buffer (approximately 0.1 m, pH 7.1) containing 15% sodium dodecyl sulfate; this solution was added to the solution containing 1% agarose as the gel apparatus. The dimensions of the gels were 90 × 6 mm. The particular samples were concentrated for electrophoresis by sedimentation at 100,000 × g for 90 min; the soluble fraction was precipitated by adding trichloroacetic acid to a final concentration of 10% and pelleted in the clinical centrifuge. All sample pellets were suspended in a solution containing 4 m urea, 1% sodium dodecyl sulfate,

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1%, mersaptoethanol, and 10% glycerol and the suspensions were then placed in a boiling water bath for 5 min. Electrophoresis was at 6 ma per tube using 250 μg of the solubilized protein in a volume of 100 μl. Bromophenol blue was used as the tracking dye, and electrophoresis was continued until the dye front was about 1 cm from the bottom of the gels (approximately 8 to 9 hours). The gels were fixed overnight in 50% trichloroacetic acid and were then stained for 60 min in a solution containing 0.25%, Coomassie brilliant blue, 50% methanol, and 10% acetic acid. Gels were destained for 36 hours in a solution containing 7% acetic acid and 5% methanol. Gel scans were performed with a densitometer (Joyce, Loebi & Co., Ltd.) on photographic transparencies. Molecular weights were estimated by comparisons with standards of known molecular weight analyzed in parallel.

For counting radioactivity the stained gels were cut into 1-mm slices with about 90 slices per gel. The position of the major bands were recorded as the gels were sliced. The slicing was done by hand with a razor blade after the gels had been placed into a grooved platform containing a series of slits spaced at 1-mm intervals.

Electron Microscopy—To monitor purity, samples were prepared for electron microscopy exactly as described by Cohen and M-Govern (38). The samples were fixed with 1% osmium tetroxide and were stained with 2% uranyl acetate. For autoradiography the procedure of Gambetti et al. (9) was followed for fixation and dehydration of the samples; an N,N-L-4 emulsion was applied and processed by the method of Caro et al. (39); synaptosomes isolated from two brain preparations incubated in the presence of sucrose gradients were also processed as a control to ensure that all of the [3H]leucine soluble in trichloroacetic acid had been removed during the sample preparation. Synaptosomes were defined as membrane-enclosed profiles either containing synaptic vesicles or exhibiting a synaptic junction. Non-synaptic profiles were classified according to the presence or absence of ribosomes. These particles contained neither synaptic vesicles nor synaptic junctions. “Unidentified particles” represents fragmented material and profiles the size of synaptosomes which could not be accurately categorized according to the criteria just presented.

Materials—All chemicals were reagent grade. Substrates for enzyme assay, bovine serum albumin, and yeast soluble RNA were obtained from Sigma Chemical Co. High specific activity (30 to 50 Ci per mmol) L-[3H]leucine was obtained from New England Nuclear. Triton X-100 and Ficoll were obtained from England Nuclear. Triton X-100 and Ficoll were obtained from England Nuclear.

RESULTS
Preparation and Purity of Synaptosomes, and Synaptosomal and Other Membranes—In our previous investigation (19) we prepared synaptosomes and various membranes by centrifugation of a crude mitochondrial pellet (P2), or a lysate derived therefrom by osmotic shock, in zonal sucrose gradients. The short time elapsed since then has seen several major methodological advances: (a) the discovery that it is impossible to obtain membrane preparations relatively free of microsomal contaminants (largely originating in smooth endoplasmic reticulum and Golgi) unless the P2 fraction is first subjected to repeated washings (40-44); and (b) that discontinuous or continuous Ficoll-sucrose gradients (45-49) afford superior separations of intact synaptosomes. Application of these principles to homogenates of incubated tissue slices led to adoption of the purification scheme outlined in Fig. 1. The enzymatic properties, with emphasis on suitable positive and negative markers (33, 40-45) of the resultant fractions are summarized in Table I. By both of these criteria, as well as on the basis of extensive electron micrographs, the fractions exhibit a purity the equal of most preparations derived from fresh tissue (for a discussion and comparison of criteria see Ref. 42), and a good deal superior to those used previously (19).

Kinetics of Incorporation of [3H]Leucine into Membrane Fractions—The rate of incorporation of [3H]leucine into the various subcellular fractions isolated from incubated brain slices is shown in Fig. 2. The radioactivity in the homogenate not precipitable by trichloroacetic acid, which represents the free intracellular [3H]leucine, equilibrated in about 30 min, consistent with the results of others (19, 46, 47). In contrast, the protein-bound radioactivity in the homogenate increased in nearly linear fashion for at least 1 hour. Jones and McLain (40) have estimated the rate of incorporation of leucine into the total proteins of brain slices to be 1.9 nmol per mg per hour and using similar calculations and assumptions we obtain a rate of 1 nmol per mg per hour. The cell-soluble and microsomal fractions showed the greatest extent of incorporation, again in agreement with earlier results (12, 19, 46, 47). The subfractions isolated from P3, the crude mitochondrial pellet, showed the least amount of incorporation. The specific activity of labeled protein in the synaptosomal and derived membrane subfractions was 25% and 31% of the specific activity of the microsomal protein, respectively, after an incubation of 60 min.

Since these values are of the same magnitude as the level of microsomal contamination, estimated from either enzymatic or morphological criteria (15 to 20%), the possibility cannot be excluded that some or even most of the label in the synaptosomal and membrane fraction derived therefrom is in fact due to such contamination. Given the current state of the art, incorporation data of this type can therefore not be used to determine the origin of the proteins of the synaptic plasma membrane.

Transfer of Labeled Proteins in Absence of Further Synthesis—If the labeled protein in the synaptosomal or synaptic membrane subfractions does indeed originate in nerve endings, one possible source of supply of this material is provided by axonal transport from the neuronal cell bodies. A series of label transfer experiments was therefore performed to test this hypothesis. Inhibitors of protein synthesis were used, since these have been shown to have no effect on axonal transport both in peripheral nerve (48, 49) and cerebral cortex (50). The effect of emetine and chloramphenicol, which specifically inhibit cytoribosomal (51, 52) and mitochondrial (18, 53, 54) protein synthesis, respectively, on incorporation into the proteins of subcellular fractions are summarized in Table II. In these experiments the brain slices were preincubated for 25 min and then exposed to [3H]leucine for 1 additional hour in the presence of the inhibitor shown. The results were in good agreement with our previous observations (52). The homogenate and the microsomal and soluble fractions were inhibited more than 90% by emetine, in accord with its postulated cytoribosomal site of action. Conversely, chloramphenicol did not affect incorporation into these fractions but did inhibit both the cell mitochondrial and synaptic mitochondrial subfractions, which were the ones least inhibited by emetine. Also, the inhibitory effects of the two drugs on the mitochondrial subfractions were additive, and resulted in virtually complete inhibition. Finally, the addition of emetine converted the synaptic mitochondrial fraction from the least to the most highly labeled subtraction. Therefore, these results are consistent with the postulated specificity of chloramphenicol in inhibiting only mitochondrial protein synthesis. Label transfer experiments were next performed using these two inhibitors. The brain preparations were allowed to synthesize protein for the first 15 min in the presence of labeled precursor and then were exposed for an additional 60 min to chloramphenicol plus emet-
FIG. 1. Subcellular fractionation of brain slices. All operations were at 4°C, and all solutions contained 10 mM leucine, pH 7.4 except for the lysis solution. During lysis of the synaptosomes the pH was maintained at 8.4 by adding 0.05 M NaOH dropwise. The samples were collected from the various gradients by aspiration with a 10-cc syringe, and the italicized fractions were routinely saved for characterization. EGTA, ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid.
Enzyme distribution in brain subfractions

Enzyme assays were performed on fractions isolated from incubated brain slices. The values are expressed as relative specific activities compared to the homogenate. The values in parentheses are the absolute activities in the homogenate expressed as micromoles of substrate reacted per mg of protein per hour for monoamine oxidase was not determined. Monoamine oxidase, acid phosphatase, and ATPase were assayed at 37°; all other enzymes were assayed at room temperature.

<table>
<thead>
<tr>
<th>Enzyme Assayed</th>
<th>Homogenate</th>
<th>Microsomes</th>
<th>Cell Soluble</th>
<th>Cell mitochondria</th>
<th>Synaptosomes</th>
<th>Synaptic Membranes</th>
<th>Synaptic mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate dehydrogenase</td>
<td>32.7±6.3</td>
<td>0.387±0.032</td>
<td>2.50±0.43</td>
<td>0.583±0.029</td>
<td>0.636±0.068</td>
<td>0.085±0.014</td>
<td>0.199±0.038</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>19.5±10.7</td>
<td>0.073±0.11</td>
<td>——</td>
<td>2.75±0.30</td>
<td>0.413±0.305</td>
<td>0.312±0.074</td>
<td>4.35±0.97</td>
</tr>
<tr>
<td>Monoamine oxidase</td>
<td>1.00, n=3</td>
<td>0.223±0.051</td>
<td>——</td>
<td>2.80±0.099</td>
<td>1.40±0.090</td>
<td>1.78±0.15</td>
<td>2.69±0.23</td>
</tr>
<tr>
<td>Rotenone insensitive NADH:cytochrome c reductase</td>
<td>1.39±0.11</td>
<td>0.866±0.116</td>
<td>0.271±0.090</td>
<td>1.32±0.24</td>
<td>0.842±0.071</td>
<td>1.86±0.17</td>
<td>1.83±0.31</td>
</tr>
<tr>
<td>RNA</td>
<td>18.5±4.8</td>
<td>2.20±0.35</td>
<td>1.13±0.31</td>
<td>0.918±0.240</td>
<td>0.501±0.030</td>
<td>0.459±0.048</td>
<td>1.08±0.18</td>
</tr>
<tr>
<td>NADPH:cytochrome c reductase</td>
<td>1.286±0.50</td>
<td>1.58±0.28</td>
<td>1.28±0.36</td>
<td>0.366±0.162</td>
<td>0.262±0.090</td>
<td>1.04±0.04</td>
<td>0.799±0.250</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>0.51±0.46</td>
<td>1.37±0.21</td>
<td>1.42±0.11</td>
<td>0.892±0.075</td>
<td>1.14±0.13</td>
<td>1.51±0.15</td>
<td>0.483±0.081</td>
</tr>
<tr>
<td>(Na+ + K+)-activated ouabain-sensitive ATPase</td>
<td>10.2±2.8</td>
<td>0.732±0.248</td>
<td>——</td>
<td>0.809±0.345</td>
<td>1.48±0.24</td>
<td>4.57±0.67</td>
<td>0.466±0.190</td>
</tr>
</tbody>
</table>

Table II

Inhibition by emetine and chloramphenicol of incorporation of L-leucine into rat brain subfractions

These data are the means (standard deviations less than 5%) determined from three separate sets of experiments. All values are expressed as per cent inhibition of protein synthesis by comparison of the amount of incorporated label in the slices incubated in the control medium to the amount in the slices incubated in the same medium containing the respective drugs (ometine = 100 pg per ml; chloramphenicol = 50 pg per ml). Preincubation in medium ± drug for 25 min; incubation in the presence of [3H]-leucine for 60 min.

<table>
<thead>
<tr>
<th>Subfraction</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emetine</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Homogenate</td>
<td>94</td>
</tr>
<tr>
<td>Microsomes</td>
<td>90</td>
</tr>
<tr>
<td>Cell-soluble</td>
<td>78</td>
</tr>
<tr>
<td>Cell mitochondria</td>
<td>10</td>
</tr>
<tr>
<td>Synaptosome</td>
<td>86</td>
</tr>
<tr>
<td>Synaptic membrane</td>
<td>3.8</td>
</tr>
<tr>
<td>Synaptic mitochondria</td>
<td>59</td>
</tr>
</tbody>
</table>
was by pelleting the slices in the clinical centrifuge and resuspending in 10 ml of the pre-oxygenated medium containing 100 μg per ml of chloramphenicol and 100 μg per ml of emetine. Transfer was by pelleting the slices in the clinical centrifuge and resuspending in 10 ml of the pre-oxygenated medium containing inhibitors at 37°C all in less than 1 min. The incubation was then continued for an additional 30 or 60 min. The results are the averages of two separate experiments. Left: L, trichloroacetic acid-soluble; Δ, cell-soluble; ▲, microsomes; O, homogenate; ●, synaptosomes; X, cell mitochondria. Right: O, homogenate; ●, synaptic membranes; X, synaptic mitochondria.

FIG. 3. The effect of label transfer in the presence of emetine and chloramphenicol (CAP) on the incorporation of [3H]leucine into brain subcellular fractions. The slices were incubated in [3H]leucine (50 μCi per set) for 15 min in the control medium when they were transferred to fresh medium (arrows) containing 50 μg per ml of chloramphenicol and 100 μg per ml of emetine. Transfer was by pelleting the slices in the clinical centrifuge and resuspending in 10 ml of the pre-oxygenated medium containing inhibitors at 37°C all in less than 1 min. The incubation was then continued for an additional 30 or 60 min. The results are the averages of two separate experiments. Left: L, trichloroacetic acid-soluble; Δ, cell-soluble; ▲, microsomes; O, homogenate; ●, synaptosomes; X, cell mitochondria. Right: O, homogenate; ●, synaptic membranes; X, synaptic mitochondria.

FIG. 4. The effect of label transfer in the presence of cycloheximide on the incorporation of [3H]leucine into brain subcellular fractions. The slices were incubated exactly as described by White et al. (18) with 50 μCi of [3H]leucine per set. After 15 min of synthesis, 100 μl of a cycloheximide solution was added to the medium to a final concentration of 100 μg per ml (arrows). The incubation was then continued for 15 or 45 min. The results are the averages of 2 separate experiments. Left: Δ, cell-soluble; ▲, microsomes; O, homogenate; ●, synaptosomes; X, cell mitochondria. Right: O, homogenate; ●, synaptic membranes; X, synaptic mitochondria.

Synaptic membrane subfractions increased in specific activity during the chase, a period during which the mitochondrial subfraction increased by nearly 60%. This result confirms the existence of mitochondrial protein synthesis in situ, a process known to be insensitive to cycloheximide (18). If this supposition is correct, this incorporation should be sensitive to chloramphenicol, an expectation borne out by the results shown in Fig. 3. In contrast, under the conditions used here, we observed no increase in the radioactivity in the presence of cycloheximide in the mitochondrial subfraction derived from cell bodies.

Transfer of Labeled Proteins in Absence of Axonal Transport—As a final test whether transport of protein synthesized in the cell bodies of brain slices was responsible for the labeling of synaptosomes, the preparations were incubated in the presence of colchicine, an agent that is known to inhibit both fast and slow axonal (49, 55, 56), as well as dendritic (57), transport. A control experiment showed colchicine at a concentration of 1.0 mM to have no effect on protein synthesis in the homogenate. The time course of incorporation under these conditions is shown in Fig. 5. The effect of cycloheximide on the incorporation of [3H]leucine into brain subcellular fractions. The slices were preincubated in 1.0 mM colchicine for 25 min, and 50 μCi of [3H]leucine per set were then added for the respective incubation times also in the presence of the drug. The results are the averages of two separate experiments. The values obtained for the cell and synaptosomal mitochondrial subfractions were not significantly different. Control experiments showed colchicine to have no effect on protein synthesis in the homogenate: in 60 min the control preparations synthesized 6100 ± 1500 cpm per mg of protein and the colchicine-treated preparations synthesized 6450 ± 1230 cpm per mg of protein, with three preparations each.

Localization of Newly Synthesized Protein by Radioautography—The most direct demonstration of the reality of rapid synthesis of proteins in synaptic structures is provided by radioautography. In order to perform such experiments two brain preparations were incubated in the standard medium and two in this medium plus emetine. The extent of specific incorporation of label actually observed was 17,600 cpm per mg of synaptosomal protein. Preparations exposed to emetine served as internal controls; if the silver grains in the experimental sample are in fact due to protein synthesis, treatment with this inhibitor, which inhibited incorporation 92%, should also result in a significant reduction in silver grains. To determine the area contributed by the various particles in the synaptosomal fraction, thin sections were cut and examined with the electron microscope. The results are shown in the first column of Table III. Synaptosomes accounted for the majority of the area occupied by identifiable entities, and this area was 3 times that contributed by nonsynaptic entities. Free mitochondria and rough endoplasmic reticulum contributed only a small percentage to the total. Unidentified particles, as defined under "Experimental Procedures," accounted for approximately 40% of the total particulate area. Silver grains were counted after a 56-day exposure. Analysis of the samples isolated from the preparations incubated with emetine showed only an insignificant number of grains, indistinguishable from background. In contrast, the samples isolated from the experimental preparation exhibited extensive incorporation. However, even a cursory examination indicated that a high percentage of silver grains were concentrated, frequently in clusters, over nonsynaptic, ribosome-containing structures, many of them membrane-enclosed profiles with ribosomes in their interior. Single silver grains over synaptosomes were...
In Column C sections from the samples used in calculating areas were subjected to radioautography. The silver grains in 100 micrographs at a magnification of 25,000 X were examined. Structures were identified as described under "Experimental Procedures." Incorporation was for 75 min. with 500 Ci of [3H]leucine; emetine (100 pg per ml) was added to one set for a determination of background incorporation.

<table>
<thead>
<tr>
<th>Particle</th>
<th>A % fraction of area</th>
<th>B % fraction of identified structures</th>
<th>C Grains counted</th>
<th>C/A Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptosome with junction</td>
<td>8.9</td>
<td>17</td>
<td>20</td>
<td>2.2</td>
</tr>
<tr>
<td>Synaptosome without junction</td>
<td>18</td>
<td>51</td>
<td>14</td>
<td>0.78</td>
</tr>
<tr>
<td>Nonsynaptic process + ribosomes</td>
<td>4.0</td>
<td>4.9</td>
<td>38</td>
<td>9.5</td>
</tr>
<tr>
<td>Nonsynaptic process - ribosomes</td>
<td>4.7</td>
<td>4.9</td>
<td>10</td>
<td>2.1</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>1.4</td>
<td>11</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>Free mitochondria</td>
<td>4.7</td>
<td>11</td>
<td>9</td>
<td>1.9</td>
</tr>
<tr>
<td>Unidentified particles</td>
<td>28</td>
<td>143</td>
<td>5.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Nonparticulate space</td>
<td>30</td>
<td>16</td>
<td>0.53</td>
<td>0.53</td>
</tr>
</tbody>
</table>

much less frequent. We attempted to quantitate these results by counting a large number of grains. An estimate of the specific activity of an organelle of a given type was made by dividing the total number of silver grains over that organelle by the total area contributed by it. An estimate of the number of silver grains attributable to background was obtained by dividing the number of silver grains over the nonparticulate space by the area contributed by the nonparticulate space, which accounted for about 30% of the total area in these micrographs. The results are presented in the remaining columns of Table III. Nonsynaptic profiles and rough endoplasmic reticulum, both studded with ribosomes, showed at least 10 times the activity of background. More than 50% of the grains, however, overlay particulate material which could not be identified on the basis of its morphology. Consequently, the calculation of the specific activities might be biased by this exclusion of a substantial fraction of the product, some of which at least may well be contributed by nerve endings that have failed to retain their junction with postsynaptic attachments. However, there was no problem in identifying synaptosomes with intact junctional complexes, and any silver grains over these structures were not likely to go uncounted. These particles showed about 4 times the activity of background with 75% of the grains either postsynaptic to the synaptosomal fraction proper would then account for <30% of the total.

**Analysis by Electrophoresis of Polypeptides Under Denaturing Conditions**—To provide a final characterization of the polypeptides synthesized in the slices, the proteins of isolated subfractions were subjected to polyacrylamide gel electrophoresis under denaturing conditions. These experiments were performed for several reasons. First, it was desirable to characterize the gross composition of the subfractions in the absence of protein synthesis. Second, the soluble and microsomal fractions incorporated the majority of the label, and it was of interest to compare these two classes of rapidly synthesized proteins. Third, the products of in situ protein synthesis by mitochondria warranted a further characterization. And fourth, although contamination may contribute a major proportion of the incorporated protein in the synaptic membrane fraction, the possibility remained that a few proteins localized in synaptic membranes were rapidly synthesized in brain slices, and that these proteins could be resolved by gel electrophoresis.

The experiments were performed by first incubating two sets of brain slices in the standard medium and two in the medium with emetine added, both for 75 min. The subcellular fractions were isolated as described earlier except that the microsomal fraction was lysed in a fashion similar to that used for the synaptosomal fraction to reduce the extent of contamination by soluble components. All of the fractions were inhibited more than 90% by emetine except for the synaptic mitochondrial fraction, which incorporated about 25% of the control label under these conditions (cell body mitochondria were not analyzed in this experiment). Protein (250 µg) from the various fractions was treated with sodium dodecyl sulfate and analyzed on gels as described under "Experimental Procedures." The gels were stained with Coomassie brilliant blue and their appearance is shown in Fig. 6. Densitometric scans of the photographic transparencies are shown in Fig. 7. The staining patterns observed in the particulate fractions are quite similar to those reported by us for fresh preparations (42). Twenty or more bands could be identified on all gels although the pattern given by the synaptic mitochondria appeared less complex than any of the others.

The following data summarize the results obtained

1. All of the subfractions showed a major band, C, with a molecular weight approximately equal to 50,000.

2. The synaptic mitochondrial subfraction contained two major bands, B and F, with molecular weights approximately 55,000 and 32,000, respectively, not prominent in any of the other fractions.

3. The soluble fraction contained two characteristic bands, D and G, with molecular weights of approximately 42,000 and 27,000, respectively, not prominent in any of the other subfractions.

4. The synaptic membrane and the microsomal subfractions contained a characteristic major band, A, of molecular weight approximately 92,000, absent from the other fractions.

5. The patterns exhibited by the synaptic membrane and microsomal fractions were very similar. The synaptic membranes contained two minor components, E and F, which were substantially reduced in intensity relative to the bands of identical mobility also found in the microsomal, and synaptic mitochondrial fractions, respectively.

To examine the protein labeling pattern in the various subfractions, duplicate gels were cut into 1-mm slices and their radioactivity in the presence and absence of emetine was determined (cf. "Experimental Procedures"). The data summarized in Fig. 8 lead to the following conclusions.

1. The samples isolated from the preparations exposed to emetine showed no significant labeling along the gels, except for the synaptic mitochondria.
2. The soluble fraction showed the highest specific activity and a broad distribution of labeled proteins throughout the molecular weight range examined. Relative to those in the microsomal fraction, the proteins of the soluble fraction contained a greater fraction of label in proteins of molecular weight less than 50,000. Some proteins were probably synthesized more rapidly or at least exhibited a higher specific activity than others. For example, Band C, which exhibited the greatest staining intensity and was probably the predominant species, contained no more label than proteins of higher molecular weight with a much less prominent intensity.

3. In the gels derived from both the synaptic membrane and microsomal fractions a labeling peak was observed in a region corresponding to a somewhat smaller size than Peak C. In the higher molecular weight regions between the origin and Band C, no convincing differences could be found in the labeling between these two subfractions. However, relative to staining intensity the synaptic membrane subfraction did contain more label in the region of Bands E and F than did the microsomal fraction.

4. In contrast to all other fractions, the newly synthesized proteins in the synaptic mitochondria appeared to be predominantly of molecular weight \( \leq 50,000 \). It was also apparent that the major bands, B, C, and F in the synaptic mitochondria, were not labeled nearly in proportion to their staining intensities. The emetine-treated samples showed no significant label in proteins of molecular weight greater than 50,000, and the label appeared to be concentrated in a major peak with an approximate molecular weight of 38,000 and two minor peaks with molecular weights around 26,000 and 20,000 (called Peaks 1, 2, and 3, respectively, in the emetine trace). Since there was no prominent staining band in this region of the gel the major peak of molecular weight 38,000 probably represents protein(s) synthesized to a greater extent than the other mitochondrial proteins.

**DISCUSSION**

**Purity of Membrane Fractions**—As described in the introduction considerable effort was expended in improving the methods for the separation of various intracellular, and limiting, membranes so as to obtain labeled synaptic plasma membranes in as pure a form as possible. It is evident that all conclusions dealing with rates and extent of labeling of such entities must ultimately hinge on the absence of more highly labeled contaminants in the fraction of origin. However, with the current state of the art, even for the best preparations, including the ones described here, it is not possible to obtain synaptic membranes in reasonable yield that are not contaminated by microsomal membranes to the extent of 10 to 20%. Considering the much more rapid rate of incorporation into the proteins of that subcellular fraction, this level of contamination is quite sufficient to invalidate, or at least raise serious doubts concerning, any conclusions based on incorporation data alone. It is for this reason that we have attempted to buttress our conclusion that there is some limited synthesis of the proteins in the synaptic membranes in tissue slices in vitro by several additional lines of experimentation.

**Modes and Routes of Synthesis**—Given the possibility that slices of cerebral cortex are capable of providing for the synthesis of some membrane proteins, although at a low level, the question arises as to the mode, site, and possible transfer of these newly made polypeptides. Regarding mode, most investigators are now in agreement that only two merit serious consideration, namely those performed by polyribosomes of the cytosolic or the mitochondrial variety. The following possibilities concerning
site and transport of polypeptides synthesized by these systems are likely a priori.

1. Transport of newly synthesized protein from neuronal cell bodies along axons to presynaptic nerve terminals. Rates of axonal transport as high as 10 mm per hour have been recorded in both peripheral nerve and the CNS (58, 59). At this rate proteins could traverse almost the entire length of the cortex in 1 hour, or a slice of the dimensions used in considerably less time than this.

2. In situ synthesis by presynaptic nerve terminals. Although no ribosomes have been seen in these structures there have been recurring claims that not only their mitochondria but they themselves are capable of limiting protein synthesis, the products of which may be transferred to the membrane (9, 15).

3. In situ synthesis by postsynaptic entities still attached to synaptosomes. Ribosomes are occasionally observed in the postsynaptic portion of synaptic complexes, especially in axosomal and axo-dendritic synapses impinging on the larger dendrites.

In addition, we performed a number of experiments designed to test the hypothesis, considered plausible on the basis of earlier studies from this laboratory (19), that System 1 makes a significant contribution to the synthesis of synaptic membranes in tissue slices. The experiments were of two types: the first used inhibitors of protein synthesis to detect transfer of label between the sites of synthesis and utilization; the second used colchicine to interrupt axonal transport. The results obtained were negative and, provided that colchicine can penetrate and is active in slices, they therefore exclude this mechanism as a significant source of newly formed protein. These conclusions are opposite from a suggestion made earlier by some of us (19). We have no ready explanation for this discrepancy. What is certain is that the present system constitutes a significant improvement in purity. The membranes found to be labeled earlier in cycloheximide transfer experiments may therefore either not be synaptic in origin at all, or may be representative of a fraction that is not collected in our current synaptic plasma membrane fraction. Furthermore, the likelihood of axonal transport making a significant contribution during the incubation period is considered unlikely according to the results of Droz (5) and Zatz and Barondes (60), which show that the label reaches nerve endings only after much longer times. The experiments with emetine and chloramphenicol, particularly in conjunction with the results of gel electrophoresis (next section), also serve to rule out the remote possibility that synaptic mitochondria make some proteins destined for export to other membranous organelles of the synapse.

Quantitative radioautography was used to determine whether System 2 or 3 was responsible for the observed incorporation. The conclusions from these experiments appear relatively straightforward, except for the possibility that we have failed to recognize some of the labeled synaptosomes because they no longer retain their distinguishing features (presence of storage vesicles or junctional complexes). Most of the label can be accounted for by two structures: by a contaminant consisting of membranous vesicles studded with ribosomes analogous to the structures previously described by Morgan (61), which accounts for the bulk of the observed radioactivity (9, 15); and by synaptosomes with intact junctional complexes, with the label concentrated at, or on, the postsynaptic side of the junction. This conclusion appears to be at variance with some earlier studies using the same technique which purported to show local protein synthesis in presynaptic membranes at a low but significant level (9, 15). Although one of these reports (9) did eliminate the possibility of mitochondrial contributions by the use of chloramphenicol, none of them employed inhibitors of cytoplasmic protein synthesis to ascertain whether the observed labeling was in fact due to the process under investigation. In fact, Whittaker (16) has demonstrated that this type of incorporation is absent in puromycin-treated controls and can therefore not be ascribed to normal protein synthesis. The absence of a protein-synthesizing system at this location is also confirmed by the extremely low and insignificant levels of rRNA intrinsic to the synaptic membrane (40, 42, 45), all of which appears to be of the mitochondrial variety.

In view of these facts we are puzzled by two recent, mutually contradictory reports which claim that isolated synaptic plasma membranes are capable of substantial levels of protein synthesis (62, 63). In the studies by Gilbert (62) the incorporation reaction was shown to be only partially sensitive to cycloheximide (56%), and insensitive to chloramphenicol, whereas in those of Ramirez et al. (63) the specificity was reversed, with complete insensitivity to the first inhibitor and complete sensitivity to the second inhibitor. The amount of RNA associated with the membranes was variously reported as 23 µg (62) and 6 µg (63) per mg of protein. The second study furthermore reports a final yield of synaptic membrane of 5 mg of protein per g wet weight of brain. This value exceeds by a factor of 10 the estimate of the total amount of synaptic membranes in rat cerebral cortex (42) and is only slightly lower than the total neuronal plasma membrane in this tissue estimated as 90 mg per g by Morgan et al. (40).

**Nature of Polypeptide Synthesized**—We had hoped that resolution of polypeptides by electrophoresis under dissociating conditions would permit us to draw hard inferences concerning differences between various membrane fractions. Unfortunately, due to the now well established (42, 43, 64, 65) similarity in the qualitative, and for most species even quantitative, distribution of molecular weights of the polypeptides found in various limiting and intracellular membranes, this expectation was not fully realized. However, we did detect a persistent and reproducible difference in the labeling patterns, with a peak in regions E and F of gels of the synaptic membrane fraction that was absent from the microsomes. Until the nature of the polypeptides responsible can be established the possibility exists that they are contributed not by the synaptic membrane (presumably by its postsynaptic portion), but by a different membranous entity found in the same fraction, perhaps by the particular form of the rough endoplasm reticulum now known to be responsible for the incorporation of much of the label.

Due to their distinctive patterns, much firmer conclusions can be drawn with regard to protein synthesis by mitochondria. Surprisingly, in these tissue slices only the organelles of the synapto-
the patterns of labeled polypeptides (which appear similar to but are perhaps more manageable than those of other mammalian systems (88)) suggest that this system has certain advantages for studies of the products of mitochondrial protein synthesis.

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Fig. 8. Polycataylamine gel electrophoresis of newly synthesized proteins in brain subcellular fractions. The gels depicted in Fig. 6 were sliced and counted as described under "Experimental Procedures." Duplicate gels from both the emetine-treated and the untreated control samples were counted in each fraction. The upper tracing in each plot is that of a representative gel from the untreated control and the lower tracing is that of a representative gel from the emetine-treated sample. The letters in brackets show the slice numbers of the major stained bands. Peaks I, 2, and 3 is the emetine tracing in the synaptic mitochondrial fraction are described in the text. Protein (250 µg) in a volume of 100 µl was applied to all gels.
Synthesis of membrane protein in slices of rat cerebral cortex.
L R Jones, H R Mahler and W J Moore


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