Evidence for Protein Synthesis in Synaptosomal Membranes*

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SUMMARY

Evidence is presented that a brain fraction containing synaptosomal membrane fragments and ghosts prepared from lysed rat forebrain synaptosomes synthesizes protein in a cell free system. The protein synthesis was dependent upon the addition of a synaptosomal membrane fraction, Mg++, monovalent cations, ATP, GTP, and enzyme factors prepared from intact synaptosomes. Protein synthesis was inhibited by ribonuclease, cyclohexamide, puromycin, and phosphate ion, but chloramphenicol, erythromycin, ouabain, and KCN had no significant effect. The products of protein synthesis with both the synaptosomal membrane fraction and microsomal fractions from rat forebrain were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The distribution of the synaptosomal membrane fraction product in the polyacrylamide gels differed significantly from the microsomal product. Also the distribution of products following density gradient centrifugation and a comparison of requirements for monovalent cations further differentiated protein synthesis with the synaptosomal membrane fraction from that with microsomal preparations.

Synaptosomes appear to be presynaptic nerve endings which detach from their axons and postsynaptic attachments and seal off to form small particles when brain tissue is homogenized (1, 2). These particles contain mitochondria and synaptic vesicles and retain many of the morphological and chemical characteristics of the intact synaptic terminal. Several aspects of neuronal function have been studied with synaptosomes including protein synthesis (3–6). Observations indicate that protein synthesis may occur in at least two locations within synaptosomes; in mitochondria and in another system (4, 7) which is sensitive to cycloheximide inhibition. Work in several laboratories (3, 8) indicates that possible locations for extra-mitochondrial synaptosomal protein synthesis may include synaptosomal cytoplasm and the synaptosomal outer membrane which frequently contain fragments of postsynaptic membrane. Consistent with the possibility of synaptic membrane protein synthesis are findings that some of the protein synthesized within intact synaptosomes is found within the synaptosomal membranes and that these membranes contain RNA (8). However, Morgan (9) has proposed that cycloheximide-sensitive protein synthesis in synaptosomal systems may be due to contamination by a ribosome containing particle which contains soluble enzymes and is limited by a plasma membrane.

This paper will present evidence that protein synthesis occurs in a tissue fraction which contains synaptosomal membranes and ghosts prepared from lysed rat forebrain synaptosomes. The membranes were separated from microsomes, synaptic vesicles, and mitochondria by ultracentrifugation on a discontinuous sucrose gradient. Studies were done to differentiate the characteristics of this system from protein synthesis in microsomal, mitochondrial, and intact synaptosomal fractions.

EXPERIMENTAL PROCEDURES

Materials—Uniformly labeled L-[4,5-3H]leucine, uniformly labeled L-[3H]valine, and L-[4,5,6-3H]leucine were obtained from Amer sham-Searle; diithiothreitol, phosphoenehydroxyurate, phosphoenolpyruvate kinase, antimycin A, ouabain, chloramphenicol, cycloheximide, 35Cl-L-amino acids and DEAE-cellulose (Cellex D, Bio-Rad) from Calbiochem; ATP, GTP, yeast RNA, salmon DNA, cytochrome c (horse heart), erythromycin, vitamin B12, folie acid, and phenylhydrazine hydrochloride from Sigma; 1-fluoro-2,4-dinitrobenzene, serylamide, methylleucis sarcosyiamide, 2-mercaptoethanol, pyruvate Y, and tetramethylethylenediamine from Eastman; sucrose (ultrapure), Triton X-100, and sodium dodecyl sulfate from Schwarz-Mann; sodium dodecyl sulfate from Matheson, Coleman and Bell; ribonuclease A (phosphate free) and deoxyribonuclease from Worthington; and paraaminodihydrochloride from Nutritional Biochemicals.

Preparation of P2 Fraction and Rat Forebrain Synaptosomal Membrane Fraction—The synaptosomal membrane fraction which includes synaptosome ghosts and possibly postsynaptic membranes was prepared by a modification of the procedure of Whitaker et al. (10). Male Sprague Dawley rats, 17 days old, were killed by decapitation and their forebrains were removed. The forebrain was considered to be all tissue rostral to the colliculi and cerebellum. All subsequent procedures were carried out at 0–4°C. Each forebrain weighed about 1 g and was homogenized with three up and down strokes in 9.0 ml of 0.32 M sucrose with a Potter Elvejhem tissue grinder modified to produce a clearance between the Teflon pestle and glass tube of 0.250

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SW27 centrifuge rotor contained the following amounts and poured and allowed to equilibrate at room temperature for 20 and layered on a discontinuous sucrose gradient which had been added after the addition of standard buffer, 30 min, the suspension was centrifuged 9000 x g for 15 min to sediment unlysed synaptosomes and mitochondria. The supernatant was removed and layered on a discontinuous sucrose gradient which had been poured and allowed to equilibrate at room temperature for 20 min before cooling to ²C. Each gradient tube for a Beckman SW27 centrifuge rotor contained the following amounts and molarities of sucrose from bottom to top: 12 ml of 1.0 M; 6 ml of 0.8 M; 6 ml of 0.6 M; 6 ml of 0.4 M; and about 10 ml of sample.

All sucrose solutions were made in standard buffer. The gradients were centrifuged at 95,000 x g for 90 min. The second method started with material obtained from the method of Zomzely et al. (13), modified by adding 1 mM dithiothreitol and 0.1 mM sodium EDTA, pH 7.5, was used for preparative sucrose gradient described in the synaptosomal membrane and microsome samples. After electrophoresis the gels were sliced with a vertical Canaco gel slicer giving pieces 1.5 mm thick. Each fraction was counted in Amersham-Searle NCS tissue solubilizer and ammonia as described by Ward et al. (17) and counted by scintillation spectrometry. Counting efficiency for double label experiments was 59% for ¹⁴C and 29% for ³H with a spillover of ²° into the ³H channel of less than 0.1% and of ³H into the ¹⁴C channel of less than 0.1%. The sample was then centrifuged 100,000 x g for 30 min (about, 10% of the radioactivity was sedimented for synaptosomal membrane and microsome samples). After electrophoresis the gels were sliced with a vertical Canaco gel slicer giving pieces 1.5 mm thick. Each fraction was counted in Amersham-Searle NCS tissue solubilizer and ammonia as described by Ward et al. (17) and counted by scintillation spectrometry. Counting efficiency for double label experiments was 59% for ¹⁴C and 29% for ³H with a spillover of ²° into the ³H channel of less than 0.1% and of ³H into the ¹⁴C channel of less than 0.1%. The sample was then centrifuged 100,000 x g for 30 min (about, 10% of the radioactivity was sedimented for synaptosomal membrane and microsome samples). After electrophoresis the gels were sliced with a vertical Canaco gel slicer giving pieces 1.5 mm thick. Each fraction was counted in Amersham-Searle NCS tissue solubilizer and ammonia as described by Ward et al. (17) and counted by scintillation spectrometry. Counting efficiency for double label experiments was 59% for ¹⁴C and 29% for ³H with a spillover of ²° into the ³H channel of less than 0.1% and of ³H into the ¹⁴C channel of less than 0.1%. The sample was then centrifuged 100,000 x g for 30 min (about, 10% of the radioactivity was sedimented for synaptosomal membrane and microsome samples). After electrophoresis the gels were sliced with a vertical Canaco gel slicer giving pieces 1.5 mm thick. Each fraction was counted in Amersham-Searle NCS tissue solubilizer and ammonia as described by Ward et al. (17) and counted by scintillation spectrometry. Counting efficiency for double label experiments was 59% for ¹⁴C and 29% for ³H with a spillover of ²° into the ³H channel of less than 0.1% and of ³H into the ¹⁴C channel of less than 0.1%.

Preparation of Rat Forebrain Mitochondria—Rat forebrain mitochondria, free of intact synaptosomes were prepared by the method of Gray and Whittaker (14).

Preparation of Rabbit Retinolucye DNA, and Uniformly Labeled Hemoglobin—Procedures for the preparation of these materials have been described previously (15).

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate—
The procedure of Fairbanks (16) was used for polyacrylamide gel electrophoresis in sodium dodecyl sulfate of various protein products. Unless otherwise stated in figure legends, each gel was composed of 6.5% acrylamide, 0.2% methylenebisacrylamide, 0.5% (w/v) sodium dodecyl sulfate (obtained from Schwarz-Mann) and was buffered with 0.04 M Tris base, 0.02 M sodium acetate, 2 mM EDTA, and enough acetic acid to obtain pH 7.4. Polymerization was initiated with potassium persulfate 1.15% (w/v) and accelerated with tetramethylethylenediamine (30 µl/100 ml). The upper and lower buffer consisted of the gel Tris-acetate buffer, pH 7.4, and 1% (w/v) sodium dodecyl sulfate (obtained from Matheson). The tracking dye was pyronin Y. Electrophoresis was carried out at 20° at 5 ma per gel.

Each sample was prepared for electrophoresis by dialysis at ²C against 1 liter of 12 mM sodium bicarbonate and 5 mM 2-mercaptoethanol for 24 hours with changes of buffer every 8 hours. After dialysis the sample was incubated at 37° for 15 min in the presence of 1% (w/v) sodium dodecyl sulfate (obtained from Schwarz-Mann) and 1 mM sodium EDTA, pH 7.5. The sample was then centrifuged 100,000 x g for 30 min (about, 10% of the radioactivity was sedimented for synaptosomal membrane and microsome samples). After electrophoresis the gels were sliced with a vertical Canaco gel slicer giving pieces 1.5 mm thick. Each fraction was counted in Amersham-Searle NCS tissue solubilizer and ammonia as described by Ward et al. (17) and counted by scintillation spectrometry. Counting efficiency for double label experiments was 59% for ¹⁴C and 29% for ³H with a spillover of ²° into the ³H channel of less than 0.1% and of ³H into the ¹⁴C channel of less than 0.1%.

NH₂-terminal Leucine Analysis of Product—NH₂-terminal analysis of the product of synaptosomal membrane fraction synthesis was done by the method of Bishop et al. (18). The analyzed reaction mixture of 0.5 ml contained 0.35 mg of synaptosomal membrane fraction protein and 1.5 mg of pH 5 enzyme fraction protein plus other components listed under assay conditions below including L-[³H]leucine, specific activity 4750 mCi per mmole, and was incubated for 30 min. The derivatives were extracted into an ether phase after acid hydrolysis while unsubstituted amino acids remained in the aqueous phase. Aliquots of each phase were counted in scintillation solution containing Triton X-100 (1 liter of Triton X-100, obtained from Packard, 2 liters of toluene, and 165 ml of Liqui-flour) with an efficiency of 40% for ³H. The percentage of NH₂-terminal leucine was calculated by dividing counts per min in the ether phase by counts per min in the ether plus water phases. Analysis of rabbit hemoglobin uniformly labeled with
l-[H]leucine gave a percentage of NH₂-terminal of 0.4% (theoretical value is 0%).

**Electron Microscopy**—Membrane pellets were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.5. They were then washed in 0.1 M sodium cacodylate buffer three times and postfixed in Dalton's chrome-osmium (19). The membranes were dehydrated in ethanol and propylene oxide and embedded in Epon-Araldite. The embedded membrane pellet was cut with an LKB ultramicrotome with a diamond knife. The sections were stained with lead citrate and uranyl acetate, and subsequently were examined with a Phillips EM 200 electron microscope.

**Protein Synthesis Assay Conditions**—Each assay mixture contained the following in 50 ml; Tris-HCl, pH 7.5, 20 mM; KCl, 120 mM; MgCl₂, 4.0 mM; ATP (neutralized to pH 7.0 with KOH before use), 1.0 mM; GTP (neutralized to pH 7.0 with KOH before use), 0.4 mM; phosphoenolpyruvate (neutralized to pH 7.0 with KOH before use), 1.5 mM; phosphoenolpyruvate kinase, 0.3 i.u.; dithiothreitol, 1.0 mM; uniformly labeled L-[14C]leucine, 0.04 μM; 19 L-[12C]amino acids, 0.08 mM each; synaptosomal membrane fraction, 0.03 mg of protein; synaptosomal pH 5 enzyme fraction, 0.1 mg of protein; and unfractionated rabbit reticulocyte tRNA, 0.03 A₂₆₀ unit. Hot trichloroacetic acid-precipitable material was collected on nitrocellulose filters as described previously (13). Each filter was counted directly in 10 ml of liquifluor in toluene in a liquid scintillation counter at an efficiency of 75% for 14C. The above general conditions were optimal for the rate of protein synthesis in synaptosomal membrane and microsome systems.

**Cytochrome Oxidase (EC 1.5.3.1)**—Cytochrome oxidase enzyme activity was assayed according to the procedure of Cooperstein and Lazarow (20).

**Acid Phosphatase (EC 3.1.5.1)**—This enzyme activity was measured at pH 5 by the method of Berthet and de Duve (23).

**Nucleic Acid Determinations**—Samples were fractionated into RNA and DNA by the method of Santen and Agranoff (24). RNA was estimated by an orcinol method (25) with yeast RNA standards and DNA by a diphenylamine method (26) with salmon DNA standards.

**RESULTS AND DISCUSSION**

**Synaptosomal Membrane Fraction Preparation and Characteristics**—The synaptosomal membrane fraction used for studies of protein synthesis was prepared by a modification of the method of Whittaker et al. (10) (see “Experimental Procedures”). In this method synaptosomes were lysed with low osmotic pressure and then sedimented by centrifugation in a discontinuous sucrose gradient from 0.4 M to 1.0 M sucrose. Observations by Whittaker et al. with electron microscopy, acetylcholine determinations and marker enzymes for mitochondria indicated the following (10): synaptic vesicles were present in the 0.4 M sucrose; microsomes, synaptic vesicles, and heterogeneous membrane structures, possibly postsynaptic membranes, were found at the 0.4 M to 0.6 M sucrose interface; membranes and synaptosomal ghosts were noted at the 0.8 M to 1.0 M sucrose interface; and mitochondria and unlysed synaptosomes sedimented to the bottom of the gradient.

Electron micrographs of the synaptosomal membrane fraction used in the present work were prepared (Fig. 1). The large almost empty round membranes are probably synaptosomal ghosts. A few synaptic vesicles, but no mitochondria were noted; furthermore, cytochrome oxidase activity, a mitochondrial enzyme marker, was not detectable in this fraction (Table I). Membrane fragments of all sizes were also present. Some fragments may arise from post synaptic membranes. Fig. 2 contains electron micrographs of Fraction E microsomes obtained from the 0.4 M to 0.6 M sucrose interface of the same discontinuous sucrose gradient with which the synaptosomal membranes were prepared (see “Experimental Procedures”). The micrographs show small heterogenous membrane fragments, many containing ribosomes, and also synaptic vesicles.

The synaptosomal membrane fraction contained 32 μg of RNA per mg of protein. There was no detectable DNA. Various marker enzyme specific activities were measured in this fraction as shown in Table I. Cytochrome oxidase activity is found in mitochondrial inner membranes (27), acid phosphatase in lysosomes (23) and NADH cytochrome reductase (antimycin A insensitive) in mitochondrial outer membranes (27), and microsomes (23). There is evidence that ouabain-sensitive (Na⁺ - K⁺)-activated ATPase is found in synaptosomal membranes in high specific activity (29). The synaptosomal membrane fraction in this study contained an 8-fold higher specific activity of this enzyme than the P₅ fraction.
from which the membrane fraction was prepared (see "Experimental Procedures").

The above electron microscopic and enzyme studies do not rule out the possibility that the synaptosomal membrane fraction may contain significant amounts of brain microsomes, intact synaptosomes, or membrane fragments from disrupted glial cells, axons, and dendrites.

Characteristics of Protein Synthesis with Synaptosomal Membrane Fraction—The optimal concentrations of various components required for protein synthesis with the synaptosomal membrane fraction are listed under "Experimental Procedures."

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Table II indicates that the system is dependent on Mg++, ATP, pH 5 enzyme fraction and the synaptosomal membrane fraction, and is partially dependent on K+, an ATP-generating system, amino acids, and GTP. The time course of protein synthesis in this system was found to be linear for 10 min (Fig. 3). The rate of protein synthesis was 5.1 pmole of L-[^14C]-

Table II
Dependence of protein synthesis on various components in complete system

<table>
<thead>
<tr>
<th>Contents of reaction mixture</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>0</td>
</tr>
<tr>
<td>Minus Tris-HCl</td>
<td>5</td>
</tr>
<tr>
<td>Minus KCl</td>
<td>75</td>
</tr>
<tr>
<td>Minus MgCl₂</td>
<td>95</td>
</tr>
<tr>
<td>Minus dithiothreitol</td>
<td>0</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>96</td>
</tr>
<tr>
<td>Minus GTP</td>
<td>20</td>
</tr>
<tr>
<td>Minus phosphoenolpyruvate, phosphoenolpyruvate kinase</td>
<td>48</td>
</tr>
<tr>
<td>Minus 19 L-[^14C] amino acids</td>
<td>50</td>
</tr>
<tr>
<td>Minus tRNA</td>
<td>15</td>
</tr>
<tr>
<td>Minus pH 5 enzyme fraction</td>
<td>95</td>
</tr>
<tr>
<td>Minus synaptosomal membrane fraction</td>
<td>99</td>
</tr>
</tbody>
</table>

Table I
Comparison of enzyme-specific activities in various fractions

All data are given as micromoles per min per mg of protein. Fractions were prepared and enzyme activities measured as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DNP-sensitive (Na+−K+)-ATPase</th>
<th>Aminase A insensitive NADH oxidase</th>
<th>Cytosome oxidase</th>
<th>Acid phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₁ (synaptosomes and mitochondria)</td>
<td>0.046</td>
<td>6.1</td>
<td>0.51</td>
<td>0.0180</td>
</tr>
<tr>
<td>Synaptosomal membrane</td>
<td>0.365</td>
<td>4.2</td>
<td>0.005</td>
<td>0.0013</td>
</tr>
<tr>
<td>Fraction E microsomes</td>
<td>0.000</td>
<td>32</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Fraction G microsomes</td>
<td>0.022</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Electron micrographs of Fraction E microsomes prepared as described under "Experimental Procedures." A, magnification X 46,000. B, magnification X 82,000.

Fig. 3. [^14C]Leucine incorporation into protein as a function of time of incubation at 37°. A 30-μl aliquot was removed at each time point from a 1-ml reaction mixture which contained 0.6 mg of synaptosomal membrane fraction protein, 2 μg of pH 5 enzyme fraction protein, L-[^14C]leucine, specific activity 331 mCi per mmole, and other components as described under "Experimental Procedures."
leucine incorporated into protein per min per mg of synaptosomal membrane fraction protein. The rate with L-[\textsuperscript{14}C]valine was 4.1 pmoles per min per mg of synaptosomal membrane fraction protein. The rate of protein synthesis with L-[\textsuperscript{14}C]leucine is about 7-fold higher than the rate reported in intact synaptosomes (3). The significance of this rate increment is difficult to ascertain due to the difference in preparations (membrane fragments versus a membrane enclosed system containing a pool of unlabeled amino acids and requiring substrate transport) and differences in the assay components and requirements for protein synthesis. The dependency on the pH 5 enzyme preparation is shown in Fig. 4. The monovalent cation (K\textsuperscript{+} or NH\textsubscript{4}\textsuperscript{+}) optima of the system was 120 mM as shown in Fig. 5. Sodium ion was inhibitory at concentrations over 120 mM (Fig. 5). The Mg\textsuperscript{2+} optima was 4.0 mM and Ca\textsuperscript{2+} had very little effect on the system (Fig. 6). The dependence on added synaptosomal membrane fraction is shown in Fig. 7.

The effects of various inhibitors of protein synthesis were studied (Table III). The system was inhibited by ribonuclease, puromycin, cycloheximide, and phosphate ion. There was no significant inhibition by chloramphenicol, erythromycin, ouabain, or KCN. These inhibitor effects differ from characteristics of a protein synthesis system with synaptosomal membranes described by Ramirez et al. (30). In this system, protein synthesis was 90\% inhibited by chloramphenicol and not inhibited by RNase or cycloheximide.

The synaptosomal membrane fraction was cultured on blood agar and found to contain only 2000 bacteria per ml of fraction. In the presence of chloramphenicol (50 \mu g per ml), a drug which did not inhibit protein synthesis in the synaptosomal system, the number of bacteria was markedly reduced (300 bacteria per ml).

Further characterization of the synaptosomal membrane fraction system included NH\textsubscript{2}-terminal analysis of the product. NH\textsubscript{2}-terminal amino acid analysis with 1-fluoro-2,4-dinitrophenol of the synaptosomal membrane fraction protein product was performed.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** \[\textsuperscript{14}C\]Leucine incorporation into protein as a function of pH 5 enzyme fraction added to the reaction mixture. Each 50-\mu l reaction mixture contained 0.03 mg of synaptosomal membrane fraction protein, L-[\textsuperscript{14}C]leucine, specific activity 331 mCi per mmole, and other components as described under "Experimental Procedures." Incubation was for 10 min at 37\°C. A zero time blank value of 0.10 pmole was subtracted from all values.

![Fig. 5](http://www.jbc.org/)

**Fig. 5** (left). \[\textsuperscript{14}C\]Leucine incorporation into protein as a function of monovalent cation concentration. Each 50-\mu l reaction mixture contained 0.025 mg of synaptosomal membrane fraction protein, 0.1 mg of pH 5 enzyme fraction protein, L-[\textsuperscript{14}C]leucine, specific activity 331 mCi per mmole, and other components as described under "Experimental Procedures." Incubation was for 10 min at 37\°C. A zero time blank value of 0.10 pmole was subtracted from each value.

![Fig. 6](http://www.jbc.org/)

**Fig. 6** (center). \[\textsuperscript{14}C\]Leucine incorporation into protein as a function of divalent cation concentration. Each 50-\mu l reaction mixture contained 0.03 mg of synaptosomal membrane fraction protein, 0.1 mg of pH 5 enzyme fraction protein, L-[\textsuperscript{14}C]leucine, specific activity 331 mCi per mmole, and other components as described under "Experimental Procedures." Incubation was for 10 min at 37\°C. A zero time blank value of 0.13 pmole was subtracted from each value.

![Fig. 7](http://www.jbc.org/)

**Fig. 7** (right). \[\textsuperscript{14}C\]Leucine incorporation into protein as a function of synaptosomal membrane fraction protein added. Each 50-\mu l reaction mixture contained 0.1 mg of pH 5 enzyme fraction protein, L-[\textsuperscript{14}C]leucine, specific activity 331 mCi per mmole, and other components as described under "Experimental Procedures." Incubation was for 10 min at 37\°C. A zero time blank value of 0.13 pmole was subtracted from each value.
**Table III**

Inhibition studies of synaptosomal membrane fraction protein synthesis

<table>
<thead>
<tr>
<th>Contents of reaction mixture</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus ribonuclease</td>
<td>10 µg per ml</td>
<td>100%</td>
</tr>
<tr>
<td>Plus deoxyribonuclease</td>
<td>10 µg per ml</td>
<td>2%</td>
</tr>
<tr>
<td>Plus chloramphenicol</td>
<td>50 µg per ml</td>
<td>3%</td>
</tr>
<tr>
<td>Plus puromycin</td>
<td>50 µg per ml</td>
<td>80%</td>
</tr>
<tr>
<td>Plus cycloheximide</td>
<td>50 µg per ml</td>
<td>56%</td>
</tr>
<tr>
<td>Plus erythromycin</td>
<td>0.5 mm</td>
<td>0%</td>
</tr>
<tr>
<td>Plus ouabain</td>
<td>2.0 mm</td>
<td>0%</td>
</tr>
<tr>
<td>Plus KCN</td>
<td>1.0 mm</td>
<td>3%</td>
</tr>
<tr>
<td>Plus Potassium phosphate, pH</td>
<td>7.5</td>
<td>5.0 mm</td>
</tr>
</tbody>
</table>

labeled with [3H]leucine was 7.1% (see "Experimental Procedure" for method and conditions). This NH₃-terminal value and other findings such as puromycin inhibition and partial GTP dependency are not consistent with the possibility that a direct transfer of labeled amino acid to pre-existing protein without dependency on mRNA (31) might account for radioactivity in hot trichloroacetic acid-precipitable material measured in the protein synthesis assay.

In order to better characterize the protein synthesis apparatus in the synaptosomal membrane fraction, the effect of Triton X-100 solubilization was studied. The synaptosomal membrane fraction was treated with 0.7% (w/v) Triton X-100 and centrifuged at 100,000 × g for 2 hours. The resulting pellet was suspended in standard buffer (see "Experimental Procedures") and was found to synthesize protein under standard assay conditions. The A₂₆₀ to A₂₃₀ ratio for the Triton X-100-treated material was 1.4, consistent with the possibility that this preparation contained nucleic acid. The rate of protein synthesis was 8.3 pmoles of [U-¹⁴C]leucine incorporated into protein per min per mg of Triton X-100 treated synaptosomal membrane fraction protein, or 1.5 pmoles incorporated into protein per min per A₂₆₀ unit of Triton X-100-treated synaptosomal material.

The Possibility of Mitochondrial Protein Synthesis in Synaptosomal Membrane Fraction System—Several characteristics of intact mitochondrial protein synthesis differ from the system described in this study. Protein synthesis in mitochondria is inhibited by KCN and chloramphenicol (32, 34) and is not inhibited by cycloheximide (32, 33) or phosphate ion, unlike the synaptosomal membrane system (Table III). Furthermore, intact brain mitochondrial protein synthesis is not sensitive to ribonuclease (35) and does not require supernatant enzymes (5), a requirement of the synaptosomal system (Table II). Intact mitochondria or mitochondrial ghosts were not noted in electron micrographs of the synaptosomal membrane fraction, which also did not contain significant cytochrome oxidase activity (Table I).

An experiment was done to ascertain the possibility that protein synthesis in the synaptosomal membrane fraction was due to protein synthesis in contaminating mitochondrial ghosts or fragments possibly produced by the osmotic shock step in the synaptosomal membrane fraction preparation (see "Experimental Procedures"). Rat forebrain mitochondria were prepared by the method of Gray and Whitaker (14) by differential and discontinuous sucrose gradient centrifugation. The mitochondria were suspended in hypotonic standard buffer (see "Experimental Procedures"). 3 ml per g of starting tissue, for 30 min at 2°C. These were the same conditions used to disrupt intact synaptosomes in the synaptosomal membrane fraction preparation. The mitochondrial suspension was then fractionated by centrifugation as described under "Experimental Procedures" for the synaptosomal membrane fraction preparation. The final fraction obtained from the 0.8 M to 1.0 M interface of the preparative sucrose gradient was assayed for protein synthesis under standard conditions as listed under "Experimental Procedures." There was no detectable protein synthesis with this fraction on aliquots up to 5% of the total protein collected. Thus, protein synthesis in the synaptosomal membrane fraction probably does not have a significant contribution from mitochondrial ghosts or fragments.

The Possibility of Protein Synthesis by Intact Synaptosomes in Synaptosomal Membrane Fraction System—Protein synthesis by intact synaptosomes is not dependent on pH 5 enzyme factors, ATP or Mg²⁺ (3, 4). These components were required in the synaptosomal membrane fraction system. In addition, chloramphenicol, erythromycin, ouabain, and KCN all significantly inhibit protein synthesis in intact synaptosomes (3, 4) in contrast to the membrane system. Ribonuclease and Na⁺ have different effects on these two systems; in contrast to the present system (Table II, Fig. 5) the protein synthesis in intact synaptosomes is activated by Na⁺ (100 mM) and not significantly inhibited by ribonuclease (3).

The Possibility of Microsomal Protein Synthesis in Synaptosomal Membrane Fraction System—Contamination by whole brain microsomes could account for protein synthesis in the synaptosomal membrane fraction. In order to investigate this possibility, three types of microsome fractions were prepared. One preparation was made from whole rat forebrain with differential centrifugation by the method of Zomzely et al. (13) (see "Experimental Procedures"). This preparation will be referred to in the text as rat forebrain microsomes. A second preparation (Fraction E) was obtained from the 0.4 M to 0.6 M sucrose interface of the discontinuous sucrose gradient used to prepare the synaptosomal membrane fraction. Electron micrographs of this material are shown in Fig. 2. Membranes containing ribosomes as revealed in the electron micrographs of this microsomal fraction (Fig. 2) were not found in electron micrographs of the synaptosomal membranes prepared at high magnifications (X 50,000). A third preparation of microsomes (Fraction G) was obtained by subjecting rat forebrain microsomes to hypotonic shock and then sucrose gradient centrifugation. Those microsomes sedimenting at 0.8 M to 1.0 M sucrose were collected (see "Experimental Procedures").

The optimal concentrations of each component of the reaction mixture for protein synthesis with all microsome preparations were similar to those for the synaptosomal membrane fraction system. However, the dependencies for monovalent cations were different in the synaptosomes and microsome systems. The
electrophoresis in sodium dodecyl sulfate. With double label synthesis with the synaptosomal membrane fraction and various microsomal product radioactivity. The products from protein amounts in the denser portion of the gradient than the forebrain membrane fraction protein synthesis including nascent peptide preparative sucrose gradient (0.4 M to 0.6 M) where microsomes sediment as determined by electron microscopy (Fig. 2). In the synaptosomal membrane fraction than in an area of the somal membrane fraction than in the Fraction E microsome was less than 50% of the yield of synaptosomal membrane fraction. The yield of Fraction G microsomal protein per g of starting forebrain tissue was 0.5 ml each of the following concentrations of sucrose (M): 1.4, 1.2, 1.0, 0.8, 0.6, and 0.4. Centrifugation was done for 90 min at 98,000 × g in a Beckman SW 27 rotor. After centrifugation, a needle was inserted through the bottom of the centrifuge tube and 6-ml fractions were collected by gravity. Each fraction, 0.5 ml, was analyzed for hot trichloroacetic acid-precipitable material as described under “Experimental Procedures.” Millipore filters were dissolved in 10 ml of Bray’s solution (36) and 3H and 14C radioactivity determined by scintillation spectrometry at 20% efficiency for 3H and 45% efficiency for 14C with a spillover of 14C counts into the 3H channel of 24%, and less than 0.1% spillover in the other direction.

It was found that the rate of protein synthesis per mg of Fraction E microsomal protein per g of starting forebrain tissue was less than 50% of the yield of synaptosomal membrane fraction. The monovalent cation requirement in protein synthesis with rat forebrain microsomes was similar with Fraction G microsomes and Fraction E microsomes.

The distribution of radioactive synaptosomal membrane fraction and forebrain microsomal products with sucrose gradient analysis Two reaction mixtures were incubated for 30 min at 37°C. One contained 1.1 mg of synaptosomal membrane fraction protein, 2 mg of pH 5 enzyme fraction protein, 0.04 mM L-[3-3H]leucine, specific activity 4750 mCi per mmole, and other components listed under “Experimental Procedures” in a total volume of 1.0 ml. The second reaction mixture contained 1.4 mg of rat forebrain microsomal protein, 2 mg of pH 5 enzyme fraction protein (saturating amount for the microsomal system), 0.04 mM L-[14C]leucine, specific activity 331 mCi per mmole, and other components listed under “Experimental Procedures” in a total volume of 0.5 ml. After incubation the reaction mixtures were cooled to 2°C, diluted to 9 ml with water, and then centrifuged on a sucrose gradient composed of 6 ml each of the following concentrations of sucrose (M): 1.4, 1.2, 1.0, 0.8, 0.6, and 0.4. The total 3H or 14C cpm in each fraction divided by the total 3H or 14C cpm times 100. There were recovered 55,000 3H cpm and 27,000 14C cpm.

To detect the amount of possible microsomal contamination which could contribute to protein synthesis in the present system, the preparative procedure for the synaptosomal membrane fraction was modified by omitting the lysis step. Without prior disruption of intact synaptosomes by lysis, significant protein synthesis should not be expected with the synaptosomal membrane fraction from the preparative sucrose gradient (see “Experimental Procedures”). However, omitting lysis would...

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\text{Table IV}
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<table>
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<tr>
<th>Molality sucrose</th>
<th>3H-Protein</th>
<th>14C-Protein</th>
<th>Ratio</th>
<th>3H cpm: 14C cpm</th>
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</tr>
</tbody>
</table>

* The total 3H or 14C cpm in each fraction divided by the total 3H or 14C cpm times 100. There were recovered 55,000 3H cpm and 27,000 14C cpm.
Fig. 9 (left). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of proteins and peptides synthesized by the synaptosomal membrane fraction and Fraction G microsomes. Two reaction mixtures were simultaneously incubated for 30 min at 37°. One contained 0.2 mg of Fraction G microsome protein, 0.8 mg of pH 5 enzyme protein, L-[4,5-3H]leucine, specific activity 4570 mCi per mmole, and other components as listed under "Experimental Procedures" in a total volume of 300 µl. The second reaction mixture contained 0.4 mg of synaptosomal membrane fraction protein, 1 mg of pH 5 enzyme fraction protein, L-[14C]leucine, specific activity 331 mCi per mmole, and other components as listed under "Experimental Procedures" in a total volume of 500 µl. The amount of pH 5 enzyme was saturating for the microsomal system. After incubation both reaction mixtures were combined at 2° and prepared for gel electrophoresis as described under "Experimental Procedures." The gel was composed of 8% acrylamide and 0.16% methylene bisacrylamide. Electrophoresis was from cathode (left) to anode (right). The gels were cut and radioactivity of each fraction was determined and corrected for spillover of 14C counts into the 3H channel. Each fraction represents the counts in 1.5 mm of gel of either 3H-labeled Fraction G microsomal product (O) or 14C-labeled synaptosomal membrane product (○). The front was at Fraction 31.

Fig. 10 (right). Ratios of 14C cpm (synaptosomal membrane product) to 3H cpm (Fraction G microsomal product) in each polyacrylamide gel fraction as calculated from data in Fig. 9, which represent one experiment which has been repeated twice with similar results.

not likely affect the amount of contaminating microsomes which sediment to the same sucrose density as synaptosomal ghosts and membrane fragments (0.8 M to 1.0 M sucrose interface). Lysis was omitted in the procedure by gently suspending the 10,800 X g pellet (containing intact synaptosomes and mitochondria) in standard buffer containing 150 mM NaCl, instead of just hypo-osmolar standard buffer (see "Experimental Procedures"). Further preparative centrifugation steps were done as outlined under "Experimental Procedures." The final membrane fraction from this procedure with lysis omitted contained 6% (0.03 mg) of the expected protein yield per g of starting forebrain tissue when the lysis was included, and 8% (0.21 pmole of L-[14C]leucine incorporated into protein per min) of the expected yield of total protein synthesis activity per g of starting tissue. The protein synthesis activity per mg of protein in this preparation was 7.0 pmol/min. This activity in material from the preparative procedure with lysis omitted is most likely from microsomal protein synthesis, but activity from postsynaptic membranes, plasma membrane fragments from glial cells, axons, and dendrites, or membranes from synaptosomes inadvertently disrupted by physical manipulation during the experiment cannot be ruled out. Omitting lysis in the preparative procedure did not significantly affect the total protein synthesis activity recovered in the Fraction E microsome material at the 0.4 to 0.6 M sucrose interface of the same preparative sucrose gradient used in the above experiments (see "Experimental Procedures"). This activity was found to be 95% (0.65 pmole of L-[14C]leucine incorporated into protein per min) of the expected total protein synthesis activity per g of starting tissue when lysis was not omitted. In conclusion, these experiments indicate that probably not over 8% of the protein synthesis in the synaptosomal membrane fraction was from microsomal protein synthesis.

Conclusions—Evidence is presented that protein synthesis occurs in a membrane fraction obtained from sucrose gradient fractionation of osmotically lysed rat forebrain synaptosomes. Studies have been presented to differentiate protein synthesis in this synaptosomal membrane fraction from possible contaminating protein synthesis in mitochondria, intact synaptosomes or brain microsomes. Electron microscopic and marker enzyme analysis indicated that the membrane fraction prepared in this
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Evidence for Protein Synthesis in Synaptosomal Membranes
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