# Biochemical Studies of Synapses *in Vitro*. I. Protein Synthesis*

Lucila A. Autilio, Stanley H. Appel,† Penelope Pettis, and Pier-Luigi Gambetti

ABSTRACT: Synaptosomes have been isolated in discontinuous Ficoll gradients from 18-day-old rat brain cortices. By electron microscopy and by enzyme specific activity they appear to represent relatively purified preparations. [$^{14}$C]Leucine is incorporated into protein by these nerve ending particles at a linear rate for 20 min. Such synthesis is inhibited 70–80% by puromycin and cycloheximide and 25% by chloramphenicol. The protein synthesis is not dependent upon exogenous substrates or energy supplies, including adenosine mono-, di-, and triphosphates or guanosine triphosphate. The ionic content of the medium appears critical: 100 mM sodium and 10 mM potassium result in a fourfold stimulation over control incubations without any ions. Ouabain ($5 \times 10^{-4}$ M) produces a 50% inhibition, and dinitrophenol, oligomycin, and potassium cyanide are all potent inhibitors. The electron microscopic monitoring, the requirements including inhibitors and activators and the kinetics of the protein synthesis exclude any significant contribution by bacteria or extrasynaptosomal ribosomes, microsomes, or mitochondria. The newly synthesized radioactive protein is incorporated predominantly into particulate membrane fractions, and its rate of production is modulated by ionic constituents and endogenous energy. These results suggest that brain synapses may meet their macromolecular requirements by protein synthesis *in situ* as well as depend upon migration of protein synthesized in the cell body. Such synthesis *in vitro* may serve as a useful model to assess the mechanism of neurohormone action and the influence of energy levels and ionic constituents upon macromolecular metabolic and synaptic function.

The key to the organization and function of the nervous system is given by the pattern of synaptic connections between cells, and the efficiency of such synapses in interneuronal communication. Many of the functional characteristics of synapses have been elegantly elucidated by electrophysiological techniques (Eccles, 1964). Yet our understanding is still extremely limited as to the biochemical events which may be rate limiting in the intercellular transfer of information. Proteins are obviously important in the metabolism of neurotransmitters, the structure of membranes, as well as other vital components of the chemical transmission from one cell to another. An understanding of macromolecular metabolism, therefore, becomes critical for an understanding of synaptic function.

The synapse may be considerably removed from the cell body and its nucleus. To meet its macromolecular requirements proteins must either be synthesized at the synapse or they must migrate from the cell body to the synaptic junction. Several experiments have confirmed the classical observation of Weiss and Hiscoe (1948) that cytoplasmic components may migrate down the axon (Droz and LeBlond, 1963; Lassek, 1967; Grafstein, 1967). Barondes (1964) has postulated a similar axonal flow within the central nervous system. On the other hand, there are data suggesting that proteins may be also synthesized in cytoplasmic and mitochondrial components of axons (Edstrom, 1966; Koenig, 1967); and it is possible to infer that protein synthesis occurs in synapses from the observation that brain slices incorporate radioactive amino acids into synaptosomal proteins without a time lag (Austin and Morgan, 1967).

The synaptosomal fraction of mammalian brain is a useful *in vitro* system to study synaptic function, since morphological and biochemical evidence have shown their intactness and relative purity (DeRobertis *et al.*, 1963; DeRobertis, 1967; Whittaker *et al.*, 1964).

In the present study the capacity of isolated synapses to synthesize proteins is reported. These proteins are predominantly incorporated into membranes. The system was found to be activated by ions and was dependent upon mitochondrial supply of energy. The observed protein synthesis appears to reflect the activity of both cytoplasmic and mitochondrial constituents within the synaptosome.

## Material and Methods

Ficoll was obtained from Pharmacia. Prior to use it was dissolved in a small amount of water and reprecipitated with 95% ethanol to remove the high salt content. It was then redissolved in water and lyophilized prior to use. [$^{14}$C]Leucine (251 mCi/mmole) was obtained from New England Nuclear Corp. Purified ribonuclease was obtained from Worthington Biochemicals, puromycin from Nutritional Biochemicals, cycloheximide from California Biochemicals, chloramphenicol from Parke Davis, araldite was obtained from

* From the Departments of Neurology and Pathology, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania. *Received March 21, 1968.* This work was supported in part by a grant from the National Multiple Sclerosis Society and Grants NB 05772 and NB 07872 from the U. S. Public Health Service.

† Career development awardee of the U. S. Public Health Service. Present address: Department of Medicine, Duke University Medical Center, Durham, N. C.

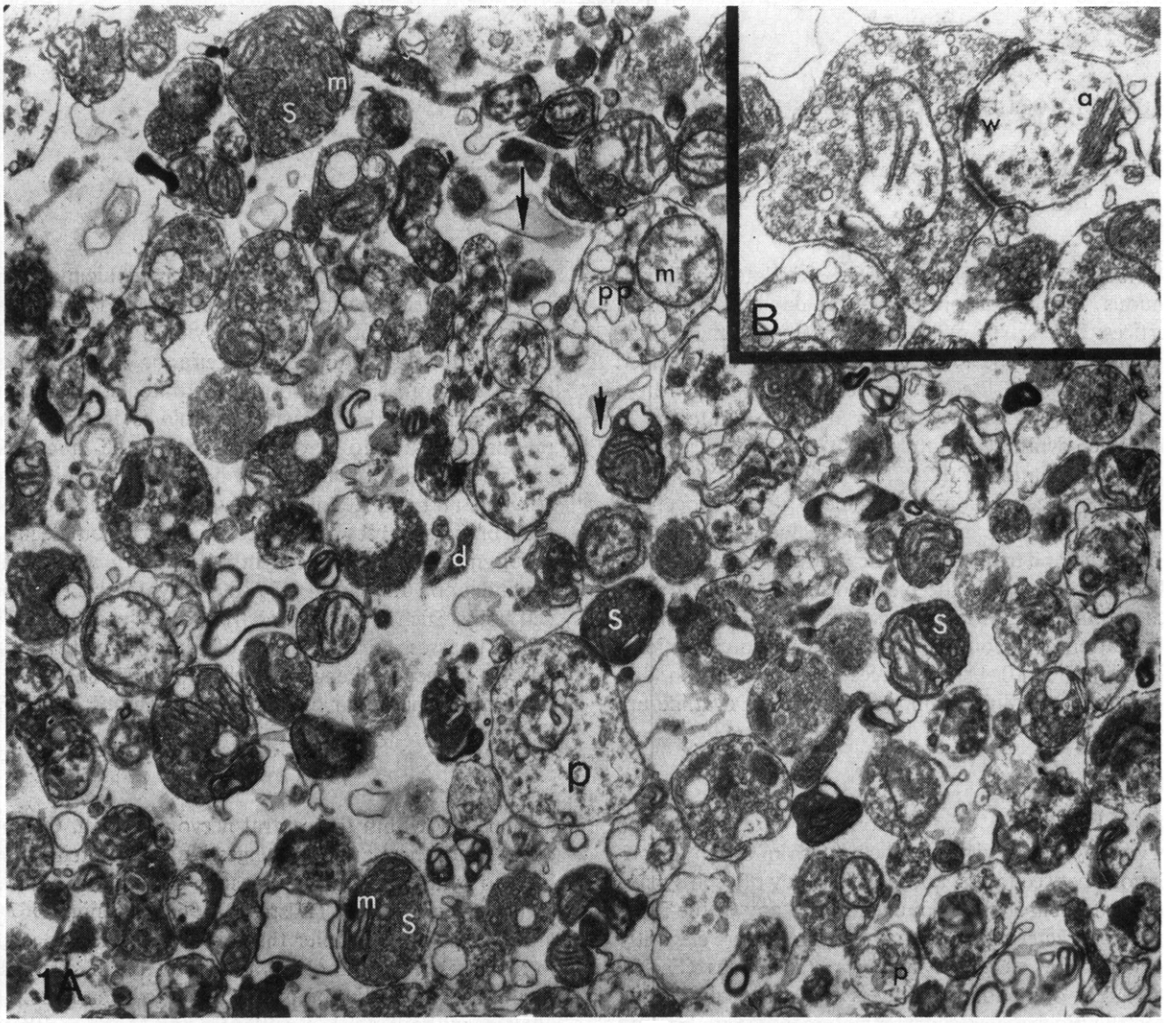


FIGURE 1: Electron micrographs of synaptosomal fraction. (A) Presynaptic endings (s) containing vesicles and occasionally mitochondria (m); p, postsynaptic component; pp, other rounded profiles, probably postsynaptic components, some of them containing mitochondria (m); arrow, membrane fragments; d, unidentifiable dense bodies ×18,000. (B) Well-preserved synaptosome; w, subsynaptic web; a, spine apparatus ×30,000.

Ciba Co., Duxford, England, oligomycin from Sigma, and ouabain from Eli Lilly and Co.

*Preparation of Synaptosomes.* Synaptosomes were prepared by a modification of the method of Abdel-Latif (1966). Rats (18-day-old) were killed by decapitation; cerebral cortices were removed rapidly and placed in cold 0.32 M sucrose. All operations were carried out at 0–4°. A 10% (w/v) homogenate in 0.32 M sucrose was prepared in a Teflon glass homogenizer (clearance 0.025 cm) with ten up-and-down strokes at 840 rpm. The nuclei and unbroken tissue fragments were separated by centrifugation at 1000*g* for 10 min. The resulting supernatant was centrifuged at 14,000*g* for 15 min to obtain a crude mitochondrial fraction which was washed once by resuspension in 0.32 M sucrose and centrifuged at 15,000*g* for 20 min. This crude mitochondrial pellet was resuspended in 0.32 M sucrose (3 ml/g of original tissue) and layered on Ficoll gradients. The gradients were prepared by layering 9.0 ml each of 20, 13, 10, 7.5, and 5% Ficoll in 0.32 M sucrose; they were allowed to equilibrate at room temperature for 1 hr and then cooled at 4° for 30 min prior to use. The gradients were centrifuged in an SW 25.2 Spinco rotor at 25,000*g* for 45 min. The two synaptosome fractions which layered between 7.5 and 13% were combined for subsequent study. They were diluted with 0.32 M sucrose and centrifuged at 40,000*g* for 30 min. The pellet was washed once in 0.32 M sucrose to remove the Ficoll. The final pellet was suspended in a loose Dounce homogenizer in 0.32 M sucrose (0.4 ml/g of original tissue) and was used for the incorporation studies.

*Enzymatic Determinations.* Succinic dehydrogenase activity was determined colorimetrically by the method of Pennington (1961). Acetylcholinesterase was determined according to Ellman *et al.* (1961) and protein by the method of Lowry *et al.* (1951).

*Electron Microscopy.* The pellets of the various fractions were fixed by suspension for 1 hr at 4° in 5% glutaraldehyde in 0.5 M cacodylate buffer (pH 7.4) and 2% 0.2 M calcium chloride (Sabatini *et al.*, 1963). After centrifugation the pellets were postfixed in 1% osmium tetroxide in buffer (Millonig, 1962) for 20 min at 4°.

The tissues were dehydrated rapidly with ethanol and embedded in araldite. Sections were cut on an LKB ultrotome with a diamond knife. Contrast was enhanced by staining with uranyl acetate and lead citrate (Millonig, 1961; Huxley and Zubay, 1961). A Siemens Elmiskop I electron microscope was used with an objective aperture of 50 $\mu$ in diameter and an accelerating current of 80 V.

*Incubation.* Unless otherwise indicated, synaptosome aliquots containing between 0.7 and 1.0 mg of protein were incubated in a Dubnoff bath shaker at 37° for 20 min. The incubation medium consisted of 0.033 M Tris-Cl buffer (pH 7.6), 0.1 M sucrose, 100 mM sodium chloride, 10 mM potassium chloride, and 1 $\mu$Ci of [$^{14}$C]-leucine in a final volume of 1.0 ml. The reaction was stopped by the addition of 5% trichloroacetic acid containing 3 mM unlabeled L-leucine.

*Preparation of Protein for Counting.* The TCA[1]-insoluble residue was dissolved in 0.25 M sodium hydroxide containing cold leucine, reprecipitated by the addition of 5% TCA, and heated twice at 90° for 10 min with 5% TCA. Lipids were extracted with warm ethanol–ether (3:1) and the precipitate was dried with ether. The pellet was dissolved in 0.2 ml of 1 N NaOH. An aliquot was dried on a filter paper disk and counted in a Packard scintillation counter in a toluene–dimethyl-POPOP–PPO mixture with an efficiency of 75%. Another aliquot was used to determine protein content.

*Separation of Labeled Synaptosome Components.* Synaptosomes were incubated as described and the incubation was stopped by addition of ice-cold 0.32 M sucrose. After centrifugation at 40,000*g* the synaptosome pellet was resuspended in water with a Dounce homogenizer (4 ml of $H_2O$/g of original tissue). After standing overnight at 4° synaptosome subfractions were prepared on a sucrose density gradient according to the technique of Whittaker *et al.* (1964). The various fractions were separated and precipitated directly by the addition of TCA to a final concentration of 5%. The precipitates were treated and counted as described above.

## Results

*Purity of the Synaptosome Fraction.* In preliminary experiments the separation of the crude mitochondrial fraction in sucrose gradients (DeRobertis *et al.*, 1963; Whittaker *et al.*, 1964) and in Ficoll gradients as described above were compared. Electron microscopic studies showed that the synaptosomal fractions prepared in the Ficoll gradient were better preserved. The distribution of enzymes was in essential agreement with those obtained by the sucrose method. Cholinesterase activity (50–60%) was associated with the nerve ending fraction. The succinic dehydrogenase activity (77–83%) was recovered in the mitochondrial fraction.

*Electron Microscopy.* Examination of the Ficoll-prepared nerve ending particles demonstrated the relative purity of the fractions. The synaptosomal fraction consisted almost entirely of membrane-limited rounded structures, 0.2–0.8 $\mu$ in diameter (Figure 1A). Approximately 60% of these structures were identified as presynaptic terminals by their content of 400–800-Å diameter vesicles and the presence of one or more mitochondria.

Membrane fragments of different size and shape were frequently seen attached to the outer membrane of the presynaptic endings, with osmiophilic material forming a synaptic cleft. Occasionally, relatively intact synaptosomes were observed (Figure 1B). In these instances the shape and size of the postsynaptic component were similar to that of the presynaptic ending. Both were membrane limited and frequently contained one or more mitochondria. The remaining rounded structures were identical with postsynaptic components and rarely contained ribosomes. In both pre- and postsynaptic structures large vesicles 0.1–0.2 $\mu$ in diameter were occasionally present, although the smaller 400–800-Å vesicles were limited to presynaptic structures. Rare mitochondria, a few unidentified dense bodies of different size and shape, membrane fragments, and vesicles were also observed as free particles (Figure 1A). Recentrifugation of the synaptosomal fraction on gradients did not enhance its purity; in fact, it markedly decreased the yield and resulted in a greater accumulation of contaminating membranes, presumably from the breakdown of previously intact nerve ending particles.

*Bacterial Contamination.* Aliquots of the nerve ending fractions plated in nutrient agar yielded approximately $10^3$ bacteria/ml. The contamination appeared to consist of a mixture of gram-positive and gram-negative bacteria with no single strain predominating. These bacteria were cultured in tryptone broth, allowed to grow to $10^9$/ml, and washed. When the rate of [$^{14}$C]-leucine incorporation into bacterial protein was assessed, a slight lag was noted followed by a linear incorporation over a period of at least 5 hr, with no activation by addition of ions. Chloramphenicol (50 $\mu$g/ml) inhibited the incorporation by bacteria approximately 95%. In addition, there was no decrease in activity when the bacteria were preincubated for 10 min at 37° or were kept overnight at 4°. Further experiments were performed in which [$^{14}$C]leucine was incorporated into bacterial protein by growth for 24 hr in tryptone broth. These bacteria were washed several times in 0.32 M sucrose until the supernatant was free of radioactivity. These labeled bacteria were added to fresh cortical tissue from which synaptosomes were subsequently isolated. Eighty per cent of the radioactivity was found in the 1000*g* nuclear fraction, and thirteen per cent in the crude mitochondrial fraction. After subfractionation in the Ficoll gradient all the crude mitochondrial radioactivity was found in the pellet. No radioactivity was present in the nerve ending fraction. These experiments, therefore, demonstrate that bacterial contribution to the amino acid incorporation of synaptosome fractions is minimal.

*In Vitro Incorporation of [$^{14}$C]Leucine into Protein.* The incorporation of [$^{14}$C]leucine into lipid-free hot TCA precipitates was relatively linear for 20 min and

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

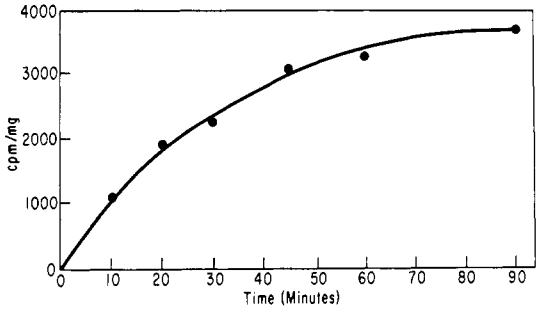


FIGURE 2: Incorporation of amino acid into protein. Incubation was at 37° for the times indicated in 1 ml containing 0.033 M Tris-Cl (pH 7.6), 0.1 M sucrose, 0.1 M NaCl, 0.01 M KCl, 1 $\mu$Ci of L-[$^{14}$C]leucine, and 0.6 mg of synaptosomal protein. The reaction was stopped at appropriate time periods by the addition of 5% trichloracetic acid and processed as in Methods.

continued for approximately 50 min (Figure 2). Preincubation of the synaptosomes for 10 min in the complete medium reduced the leucine incorporation by 20%. The synaptosomes were most active immediately after preparation and lost 80% of their activity after 24 hr at 4°. The pH optimum of the system was between pH 7.0 and 7.5. No exogenous energy source or substrate had a significant effect on the incorporation.

Maintenance of isotonicity was important for optimal rates of [$^{14}$C]leucine incorporation into protein. In the absence of sucrose or electrolytes the incorporation was 461 cpm/mg of protein, whereas in the presence of 0.3 M sucrose the incorporation increased to 761 cpm/mg of protein. Additions of ions appeared to enhance incorporation beyond that found with isotonic sucrose alone (Table I). Either sodium or potassium activates the incorporation, although sodium was always more

TABLE I: Effect of Ions and Sucrose on Synaptosomal Protein Synthesis.[a]

| Constituents | | | |
|---|---|---|---|
| Sodium (mM) | Potassium (mM) | Sucrose (M) | % of Control |
| 100 | 10 | 0.1 | 100 |
| 10 | 100 | 0.1 | 60 |
| 0 | 0 | 0.1 | 25 |
| 0 | 10 | 0.1 | 43 |
| 100 | 0 | 0.1 | 80 |
| 100 | 10 | 0.06 | 90 |
| 0 | 0 | 0.06 | 14 |
| 100 | 10 | 0.2 | 66 |
| 100 | 10 | 0.3 | 45 |
| 0 | 0 | 0.3 | 33 |

[a] Incubation was as indicated in Table III except that ions and sucrose were added in the concentrations indicated. The incorporation of 100% equals 14.0 $\mu\mu$moles of leucine incorporated/mg of protein per 20 min.

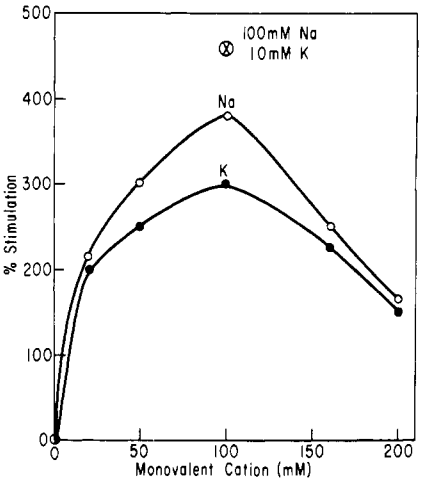


FIGURE 3: Effect of ions on synaptosomal protein synthesis. Incubations were performed as in Figure 2 in the presence of sodium or potassium ions alone at the indicated concentrations or a combination of 100 mM sodium and 10 mM potassium. The ionic activation is expressed as per cent stimulation over the control which contained no ions and 0.1 M sucrose.

effective than potassium (Figure 3). Concentrations of the individual ions greater than 150 mM were not as effectively stimulating as 100 mM. The presence of 100 mM sodium and 10 mM potassium increased the incorporation to levels greater than that observed with 110 mmoles of either ion separately. In the presence of 100 mM sodium and 10 mM potassium, 0.1 M sucrose was optimal, and increasing concentrations of sucrose were inhibitory. Sodium (10 mM) and potassium (100 mM) gave an incorporation which was 60% of that observed with 100 mM sodium and 10 mM potassium. Additions of magnesium or calcium had no activating effect. Because of the enhancement by sodium and potassium and the potential participation of a sodium-potassium-activated ATPase in our system, the effect of ouabain was studied. In Figure 4 ouabain is shown to be inhibitory with the major effect starting after 10-

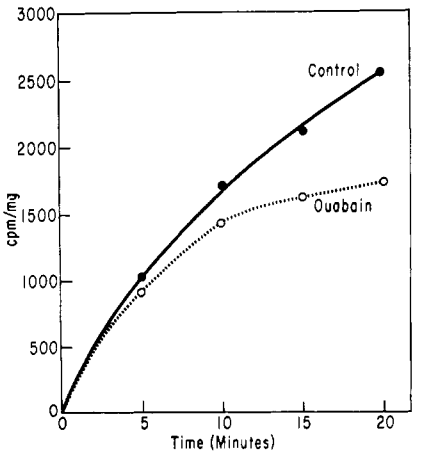


FIGURE 4: Inhibition of protein synthesis by ouabain. Ouabain was included at $5 \times 10^{-4}$ M in an incubation similar to that indicated in Figure 1 with 100 mM sodium chloride and 10 mM potassium chloride.

min incubation. Ouabain ($5 \times 10^{-4}$ M) inhibited 50% after 20-min incubation. Higher concentrations of ouabain did not significantly increase the inhibition.

*Relationship of Protein Synthesis to Age.* Synapses were prepared from rats of various ages. The incorporation of amino acid into protein was highest in synapses isolated from 8-day-old animals and continued to decrease progressively such that in the 18-day-old animals the incorporation was 33% lower and in the adult rat approximately 45% lower.

*Energy Requirements.* The addition of adenine nucleotides (AMP, ADP, or ATP) did not significantly affect the incorporation under optimal ionic concentrations (Table II). GTP at a concentration of $5 \times 10^{-3}$ M produced a 6% inhibition which is within experimental error. Furthermore, substrates which enhance oxidative phosphorylation produced either no effect or were inhibitory. α-Ketoglutarate produced no effect, whereas succinate inhibited approximately 13%, and glutamic acid inhibited 22%. Mitochondria are present within the synaptosomes as shown by electron microscopy. It thus seemed likely that the energy source, presumably ATP, was primarily derived from the energy production of mitochondria. The effect on protein synthesis of several inhibitors of mitochondrial energy metabolism was studied. Dinitrophenol at concentrations of $10^{-3}$ and $10^{-4}$ M was markedly inhibitory, as were potassium cyanide and oligomycin (Table II).

TABLE II: Effect of Energy Sources or Inhibitors on Synaptosome Protein Synthesis.[a]

| | % of Control |
|---|---|
| Complete system | 100 |
| +ATP ($10^{-4}$ M) | 100 |
| +ADP ($10^{-4}$ M) | 112 |
| +AMP ($10^{-4}$ M) | 115 |
| +Glucose ($10^{-3}$ M) | 112 |
| +Dinitrophenol ($10^{-4}$ M) | 20 |
| +KCN ($10^{-3}$ M) | 48 |
| +Oligomycin ($10^{-4}$ mg/ml) | 40 |
| +Mg ($5 \times 10^{-3}$ M) | 95 |
| +Ca ($5 \times 10^{-3}$ M) | 90 |
| +α-Ketoglutarate ($5 \times 10^{-3}$ M) | 100 |
| +Glutamate ($10^{-3}$ M) | 78 |
| +Succinate ($5 \times 10^{-3}$ M) | 87 |

[a] Incubations were as indicated in Table III with the indicated additions. The figures in parentheses represent the final concentration. 100% = 14.0 μμmoles of leucine incorporated/mg of protein per 20 min.

*Inhibitors of Protein Synthesis.* Cycloheximide inhibited our synaptosomal preparation more than 70% (Table III). Ribosomal and microsomal but not mitochondrial protein synthesis are known to be inhibited by this compound (Ennis and Lubin, 1964; Beattie *et al.*, 1967). Puromycin, which has been demonstrated to inhibit both ribosomal (Campbell *et al.*, 1966) and mitochondrial (Wheeldon and Lehninger, 1966) systems, was found to be a potent inhibitor in our system. On the other hand, chloramphenicol, which is known to inhibit mitochondrial but not ribosomal or microsomal protein synthesis (Clarke-Walker and Linnane, 1966) inhibited only 25%. It is, therefore, likely that the incorporation observed in our system is a combination of mitochondrial and extramitochondrial protein synthesis. The chloramphenicol inhibition of 25% may indicate an upper limit to the contribution of the mitochondrial protein synthesis.

To rule out the possibility of microsomes contaminating synaptosomal protein synthesis, microsomes were prepared and incubated with the synaptosomes. Under these circumstances, the incorporation of amino acid into protein was inhibited rather than enhanced. In addition, ribonuclease at concentrations known to inhibit a brain ribosomal or microsomal system (10 μg/ml) (Appel *et al.*, 1967) was found to have a minimal inhibitory effect (0–15%) (Table III).

TABLE III: Inhibitors of Synaptosomal Protein Synthesis.[a]

| | Amount | % of Control |
|---|---|---|
| Complete systems | | 100 |
| +Ribonuclease | 10 μg | 85 |
| +Chloramphenicol | 50 μg | 75 |
| +Puromycin | 1 μg | 75 |
| | 10 μg | 25 |
| | 50 μg | 12 |
| +Cycloheximide | 1 μg | 36 |
| | 10 μg | 28 |
| | 50 μg | 12 |
| +Ouabain | $5 \times 10^{-4}$ M | 50 |
| | $1 \times 10^{-3}$ M | 40 |

[a] Incubation was at 37° for 20 min in 1 ml containing 0.033 M Tris-Cl (pH 7.6), 0.1 M sucrose, 100 mM NaCl, 10 mM KCl, 1 μCi of L-[$^{14}$C]leucine, and 0.7 mg of synaptosomal protein. Incorporation with the complete system was 10.2 μμmoles of leucine/mg of protein per 20 min.

That exogenous microsomes or ribosomes do not contribute to the incorporation in our system is further indicated by the following experiment. A cell-free microsomal system was incubated with [$^{14}$C]leucine and combined with synaptosomes labeled with [$^{3}$H]leucine. This mixture was subjected to hypotonic shock according to the technique of Whittaker *et al.* (1964). It was applied to a discontinuous sucrose density gradient, and the relative distribution of the radioactive protein in the various fractions was determined. As can be seen in Table IV, the distribution of $^{14}$C- and $^{3}$H-labeled pro-

TABLE IV: Distribution of [$^3$H]Synaptosomal and [$^{14}$C]-Ribosomal Proteins After Osmotic Shock.[a]

| Sucrose Gradient (M) | Synaptosome [$^3$H]-Protein (% cpm) | Microsome [$^{14}$C]-Protein (% cpm) | Sp Act. %[$^3$H]/%[$^{14}$C] |
|---|---|---|---|
| Supernatant | 26.5 | 9.1 | 2.9 |
| 0.4 | 13.6 | 25.2 | 0.5 |
| 0.6 | 3.7 | 24.2 | 0.15 |
| 0.8 | 8.5 | 16.3 | 0.52 |
| 1.0 | 10.6 | 8.7 | 1.22 |
| 1.2 | 16.0 | 8.1 | 1.98 |
| 1.4 | 20.7 | 8.5 | 2.44 |

[a] Microsomes previously incubated with [$^{14}$C]leucine were combined with synaptosomes previously incubated with [$^3$H]leucine. The combined fractions were subjected to osmotic shock in distilled water at 4° for 15 hr. This mixture was centrifuged on a sucrose gradient and fractions corresponding to each layer were separated, precipitated with TCA, and the precipitate was washed and counted as described in Methods. The above values represent the average of three experiments. Electron microscopic monitoring of the fractions is in accord with the observations of Whittaker *et al.* (1964); 0.4 M, synaptic vesicles, microsomes; 0.6 M, microsomes, few synaptic vesicles, rare synaptosomal ghosts; 0.8 M, synaptosome ghosts, membrane fragments; 1.0 M, synaptosome ghosts, membrane fragments; 1.2 M, damaged synaptosomes; and 1.4 M, mitochondria.

teins was completely different. The proteins synthesized in the synaptosome fraction were dispersed through the gradient in soluble as well as membrane fractions, whereas the microsomal radioactive protein remained associated predominantly with the intact microsome fraction.

When synaptosomes are sonicated following incubation with labeled amino acid, the majority of radioactive proteins are present in the particulate fraction (Table V). After osmotic shock and centrifugation on a sucrose gradient, approximately 22% of the synaptosomal radioactive protein was found in the soluble fraction and 25% was associated with the mitochondria (Table VI). When these mitochondria were sonicated, all of the radioactivity was present in the particulate fraction (Table VII).

TABLE V: Distribution of Radioactivity in Sonicated Synapses.[a]

| | % cpm | % Protein | cpm/mg of Protein |
|---|---|---|---|
| Supernatant | 18.6 | 17.4 | 2860 |
| Pellet | 81.4 | 82.6 | 2621 |

[a] After a 20-min incubation with [$^{14}$C]leucine, the synaptosomes were suspended in water and sonicated for 1 min. The suspension was centrifuged at 100,000*g* for 1 hr. The supernatant was removed, the pellet was suspended, and both fractions were precipitated with 5% TCA, washed, and counted as described.

TABLE VI: Distribution of Radioactivity Following Osmotic Shock.[a]

| Sucrose Gradient (M) | % cpm | % Protein | Rel Sp Act.[b] |
|---|---|---|---|
| Supernatant | 22.9 | 24.5 | 0.93 |
| 0.4 | 12.3 | 8.9 | 1.38 |
| 0.6 | 2.9 | 2.2 | 1.31 |
| 0.8 | 3.5 | 2.0 | 1.78 |
| 1.0 | 8.8 | 7.5 | 1.17 |
| 1.2 | 24.6 | 29.0 | 0.85 |
| 1.4 | 25.0 | 25.9 | 0.97 |

[a] Synaptosomes were incubated as described in Methods. Ice-cold 0.32 M sucrose was added to stop the reaction. After centrifugation the pellet was subjected to osmotic shock overnight at 0°. The suspension was layered on a sucrose gradient and treated as described in Table IV. Each value represents the average of three experiments. [b] Relative specific activity = % cpm/% proteins.

## Discussion

The present data show that brain synaptosome preparations are capable of incorporating amino acid into protein *in vitro*. In these studies it is important to rule out contamination by bacteria, microsomes, or mitochondria that may lie outside the nerve endings. Bacterial contribution to protein synthesis was excluded by (1) the limited inhibition by chloramphenicol (25%), (2) the short period of linear protein synthesis in synaptosomes compared with the exponential rate of synthesis

TABLE VII: Distribution of Radioactivity in Sonicated Mitochondria.[a]

| | % cpm | % Protein | cpm mg of Protein |
|---|---|---|---|
| Supernatant | 1.9 | 3.5 | 1416 |
| Pellet | 98.1 | 96.5 | 2622 |

[a] The mitochondrial fraction obtained from osmotically shocked synaptosomes (Table VI) was suspended in water, sonicated, and treated as described in Table V.

in bacteria, (3) the loss of synthesizing capacity in synaptosomes kept for 24 hr at 4° and its retention with bacteria under the same circumstances, (4) the effectiveness of cycloheximide as an inhibitor despite its negligible effect on bacterial protein synthesis, and (5) the ionic activation found with synaptosomal protein synthesis but not with bacterial protein synthesis.

Similarly, a significant contribution of extra synaptosomal ribosomes or microsomes can be excluded. Ribonuclease, which is an effective inhibitor in brain cell-free ribosomal and microsomal synthesis, is ineffective in this system. Additions of supernatant enzymes, pH 5 fraction, or microsomes did not enhance the protein synthesis. Furthermore, if the protein synthesis occurs on free microsomes or ribosomes, high sodium would not be expected to activate the incorporation to the extent noted in our synaptosome preparations (Campagnoni and Mahler, 1967), and ATP would be required. In the present experiments added ATP has no effect on the incorporation.

The ionic requirements, the inhibition by ouabain, the lack of effect of exogenous substrate, and the very rare occurrence of free mitochondria by electron microscopy suggest that intrasynaptosomal mitochondria contribute to the protein synthesis.

It is unlikely that microsomes liberated during the initial homogenization of the tissue were artifactually incorporated into synaptosomal preparations. Austin and Morgan (1967) demonstrated that labeled microsomes added during homogenization of brain tissue are not incorporated into the synaptosomal fraction. In addition, the proteins synthesized in synaptosomes sedimented differently in sucrose gradients from those synthesized in microsomes.

The question arises as to what elements within the synaptosome are responsible for the observed protein synthesis. It is well known that mitochondria synthesize some of their own membrane protein (Wheeldon and Lehninger, 1966; Bachelard, 1966; Campbell *et al.*, 1966; Klee and Sokoloff, 1965; Mokrasch, 1966; Beattie *et al.*, 1967), but there is no evidence that they supply extramitochondrial proteins. Chloramphenicol, which strongly inhibits mitochondrial but not microsomal protein synthesis (Wheeldon and Lehninger, 1966), reduced the incorporation in our system by 25%. This figure compares well with the per cent of synaptosomal radioactivity found in the mitochondrial fraction after osmotic shock. Furthermore, cycloheximide, which inhibits ribosomal but not mitochondrial protein synthesis (Ennis and Lubin, 1964; Beattie *et al.*, 1967), inhibits synaptosomal activity by 70%. These results suggest that within the nerve ending particles, 75% of the radioactive protein may be synthesized outside mitochondria and 25% within mitochondria.

The synaptosomal protein synthesis appears to be independent of exogenous energy sources. ATP, ADP, and AMP had essentially no effect and $\alpha$-ketoglutarate, succinate, and glutamate inhibited rather than enhanced the incorporation. The energy for protein synthesis presumably is derived from the oxidative metabolism of intrasynaptosomal mitochondria and endogenous substrates. This is supported by the active oxygen uptake by synaptosomes and a relative lack of effect of ATP, ADP, or AMP in such experiments (A. V. Escueta and S. H. Appel, unpublished results). The inhibition by DNP and oligomycin supports the role of endogenous high-energy phosphates, and the inhibition by potassium cyanide similarly supports the role of mitochondrial function in the observed protein synthesis.

The rate of protein synthesis is linear only for 20 min and then begins to decrease. Energy availability may represent the rate-limiting factor in the over-all process, but no environmental constituents other than ions appear to enhance the level or rate of incorporation. Gradual increases in sodium appear to enhance more than potassium, but neither ion is individually as effective as the combination of 100 mM sodium and 10 mM potassium. These results suggest the participation of a sodium–potassium-activated ATPase known to be present in high concentrations in synaptosomal preparations (Hosie, 1965; Kurokawa *et al.*, 1966). Activation of the ATPase and enhancement of protein synthesis may be related, since the availability of energy *via* respiration is thought to be regulated by the sodium–potassium adenosine triphosphatase (Whittam and Blond, 1964). A more detailed investigation of the possible coupling between ATPase activity and protein synthesis in synaptosomes will be the subject of a separate report.

An additional point of interest is that the present system permits the study of purely neuronal protein synthesis, in contrast to cell-free systems from brain where no differentiation of the contribution of neuronal and glial elements can be made. Our studies do not clarify whether the amino acid incorporation into proteins occurs in the pre- or postsynaptic component. Both have mitochondria and possibly the RNA essential for protein synthesis, and only electron microscopic autoradiography may resolve the issue.

Although well established in the peripheral nerve, the existence of axonal flow has not been definitely clarified in the central nervous system, and contradictory conclusions have been reached (Barondes, 1964; Vrba, 1967). The present experiments demonstrate that isolated synapses synthesize proteins. It is, therefore, possible that *in vivo* proteins may be synthesized at nerve endings.

Of most importance are the observations that these proteins are incorporated predominantly into membrane fractions and that their rate of synthesis is modulated by ionic constituents and the availability of endogenous energy. Such synthesis appears to offer a useful model to study the way in which alterations in hormones, membrane characteristics, energy availability, and ions may affect the synthesis of membrane proteins and in turn modulate synaptic function.

While this manuscript was in preparation, Austin and Morgan (1968) reported the ability of rat cortical synaptosomes prepared in sucrose gradients to incorporate amino acids into protein. The rate of incorporation in their system is comparable with that demonstrated by us with Ficoll-prepared synaptosomes assayed in sucrose. With optimal ionic constituents, however, we note a threefold enhancement. Our data

are comparable with their data with respect to inhibition by cycloheximide and puromycin and with respect to the limited inhibition by chloramphenicol, and to the lack of effect of ribonuclease.

Acknowledgments

We are indebted to Miss Irene Evangelista for her technical assistance in morphological aspects of these studies and to Dr. Nicholas Gonatas for his helpful discussions and advice.

References

Abdel-Latif, A. A. (1966), *Biochim. Biophys. Acta 121*, 406.

Appel, S. H., Davis, W., and Scott, S. (1967), *Science 157*, 836.

Austin, L., and Morgan, I. G. (1967), *J. Neurochem. 14*, 377.

Austin, L., and Morgan, I. G. (1968), *J. Neurochem. 15*, 41.

Bachelard, H. S. (1966), *Biochem. J. 100*, 131.

Barondes, S. H. (1964), *Science 146*, 779.

Beattie, D. S., Basford, R. D., and Koritz, S. B. (1967), *Biochemistry 6*, 3099.

Campagnoni, A. T., and Mahler, A. H. (1967), *Biochemistry 6*, 956.

Campbell, L., Mahler, H., Moore, W. J., and Tewari, W. (1966), *Biochemistry 5*, 1124.

Clarke-Walker, G. D., and Linnane, A. W. (1966), *Biochem. Biophys. Res. Commun. 25*, 8.

DeRobertis, E. (1967), *Science 156*, 907.

DeRobertis, E., Rodriguez, D. L. A., Salganicoff, L., Pellegrino, D. I. A., and Zieher, L. H. (1963), *J. Neurochem. 10*, 225.

Droz, B., and LeBlond, C. P. (1963), *J. Comp. Neurol. 121*, 325.

Eccles, J. C. (1964), The Physiology of Synapses, Berlin, Springer-Verlag.

Edstrom, A. (1966), *J. Neurochem. 13*, 315.

Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. (1961), *Biochem. Pharmacol. 7*, 88.

Ennis, H. L., and Lubin, M. (1964), *Science 146*, 1474.

Grafstein, B. (1967), *Science 157*, 196.

Hosie, R. J. A. (1965), *Biochem. J. 96*, 404.

Huxley, H. E., and Zubay, G. (1961), *J. Biophys. Biochem. Cytol. 11*, 273.

Klee, C. B., and Sokoloff, L. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1014.

Koenig, E. (1967), *J. Neurochem. 14*, 437.

Kurokawa, M., Sakamato, T., and Kato, M. (1966), *Biochem. J. 97*, 833.

Lassek, R. J. (1967), *Neurosci. Res. Progr. Bull. 5*, 314.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Millonig, G. (1961), *J. Biophys. Biochem. Cytol. 11*, 736.

Millonig, G. (1962), *in* Fifth International Congress on Electron Microscopy, Breese, S. S., Jr., Ed., New York, N. Y., Academic.

Mokrasch, L. C. (1966), *J. Neurochem. 13*, 49.

Pennington, R. J. (1961), *Biochem. J. 80*, 649.

Sabatini, D. D., Bensch, K., and Barnett, R. J. (1963), *J. Cell Biol. 17*, 19.

Vrba, R. (1967), *Biochem. J. 105*, 927.

Weiss, P., and Hiscoe, H. B. (1948), *J. Exptl. Zool. 107*, 315.

Wheeldon, L. W., and Lehninger, A. L. (1966), *Biochemistry 5*, 3533.

Whittaker, V. P., Michaelson, I. A., and Kirkland, R. J. A. (1964), *Biochem. J. 90*, 293.

Whittam, R., and Blond, D. M. (1964), *Biochem. J. 92*, 147.