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In Vitro Synthesis of Brain Protein. II. Properties of the Complete System*

Kurt H. Stenzel,† Ruth F. Aronson, and Albert L. Rubin

ABSTRACT: Ribosomes and microsomes prepared from rabbit brain are active in protein synthesis. The requirements for optimal incorporation are similar to those of other mammalian cell-free systems, and include adenosine 5′-triphosphate (ATP) and an ATP regenerating system, guanosine-5′-triphosphate (GTP), pH 5 fraction, potassium, and magnesium. Amino acids are required for optimal activity in the ribosomal system. Glutamic acid alone restores 90–95% of full activity in the absence of an amino acid mixture. Ribonuclease and puromycin inhibit the system, whereas deoxyribonuclease, actinomycin, and chloramphenicol do not. Although initially the postmitochondrial fractions consist almost entirely of polysomes, the final preparation used in incorporation studies consists of both single and aggregated ribosomes. The aggregates are more active than the single ribosomes in *in vitro* protein synthesis. This system provides a useful *in vitro* model for studying the products and control mechanisms of brain protein synthesis.

The role of proteins in the specific functions of nervous tissue is not known. Molecular theories of neuronal transmission and memory require that macromolecular substances subserve specific roles in either impulse propagation, synaptic transmission, or recognition of specific neuronal circuits (Schmitt, 1964). Cell-free systems derived from brain are of interest not only as models of mammalian subcellular protein synthesis, but also as possible means of investigating relationships between electrical activity in the nervous system and dynamic aspects of polypeptide synthesis. In a previous publication we reported the formation of a characteristic nervous tissue protein in cell-free systems derived from brain (Rubin and Stenzel, 1965). These systems may thus provide *in vitro* models for studying products and control mechanisms of brain protein synthesis and the relationships of these metabolic parameters to the unique properties of neurons. This report describes the characteristics of cell-free systems derived from homogenates of rabbit brain cortex.

Materials and Methods

Reagents. Inorganic reagents were commercial

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† Fellow of the New York Heart Association.

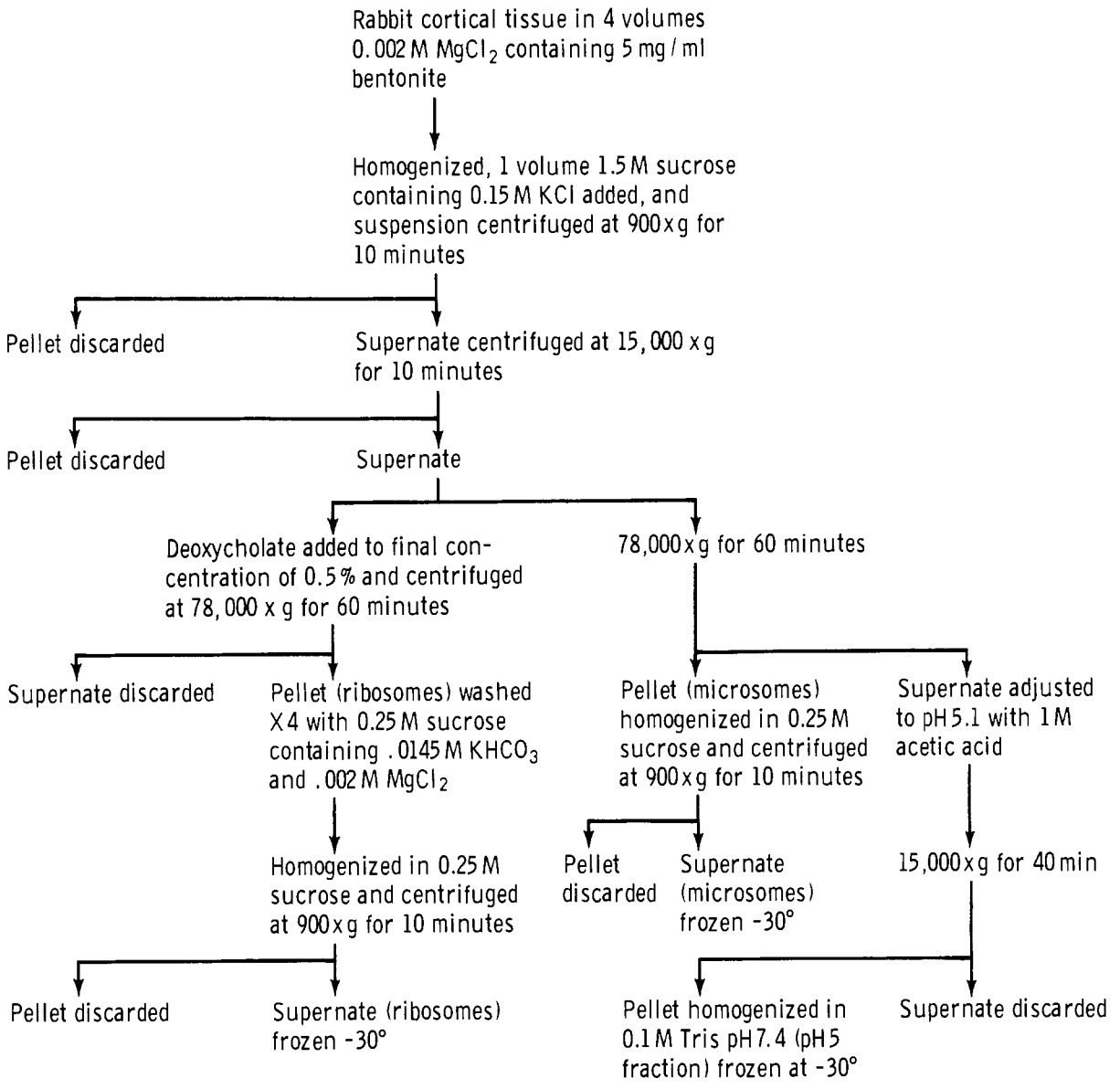

FIGURE 1: Flow sheet for the preparation of cell fractions for brain cell-free system.

reagent grade. Adenosine 5'-triphosphate (ATP)[1] and guanosine 5'-triphosphate (GTP) were obtained from Pabst Laboratories, 2-phosphoenolpyruvic acid and L-amino acids from Calbiochem, pyruvate kinase from C. F. Boehringer, reduced glutathione (GSH) from Schwarz BioResearch, Inc., sucrose and bentonite from Fisher Scientific Co., ribonuclease and deoxycholate (DOC) from Mann Research Laboratories, Tris from Sigma Chemical Co., deoxyribonuclease from Nutritional Biochemicals, and uniformly labeled [^{14}C]-leucine (233 mc/mM) from New England Nuclear Corp.

Analytical Methods. Protein determinations were made by the method of Lowry *et al.* (1951), and ribonucleic acid (RNA) determinations by a modification of the method of Mejbaum (1939). Sucrose density gradient sedimentation was done by the method of Britten and Roberts (1960) using 24 ml of 30–15% linear gradients and centrifuging for 120 min at 25,000 rpm in the SW 25 rotor of a Spinco preparative ultracentrifuge. Sucrose was prepared in 10^{-2} M Tris, pH 7.4, containing 10^{-2} M KCl and 1.5×10^{-3} M $MgCl_2$. Radioactivity was measured on 5% trichloroacetic–2.5% sodium tungstate (TCA–Tung) precipitates washed as previously described (Rubin and Stenzel, 1965) and counted in a Nuclear Chicago gas flow counter with a

[1] Abbreviations used are: ATP, adenosine 5'-triphosphate; GTP, guanosine 5'-triphosphate; GSH, reduced glutathione; DOC, sodium deoxycholate; TCA–Tung, 5% trichloroacetic acid–2.5% sodium tungstate solution.

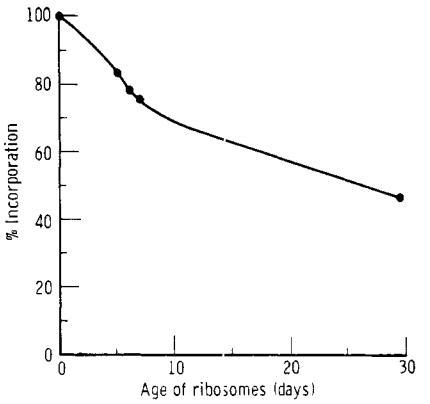

FIGURE 2: Effect of storage of ribosomes in 0.25 M sucrose at −30° on ability to incorporate [^{14}C]leucine into TCA-Tung precipitable material. 100% incorporation is the number of counts obtained in the complete system with ribosomes and pH 5 fraction prepared within 24 hr of the experiment.

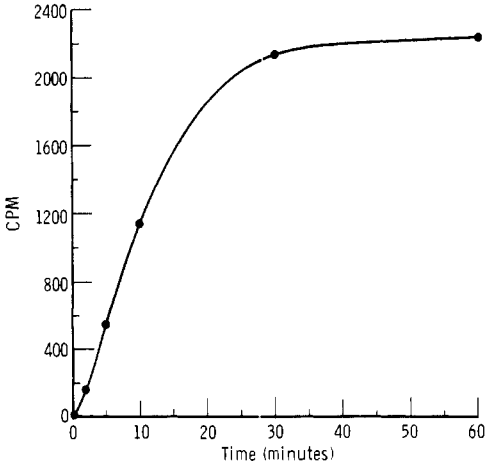

FIGURE 3: Incorporation of [^{14}C]leucine into TCA-Tung precipitable material as a function of time in the complete ribosomal brain cell-free system.

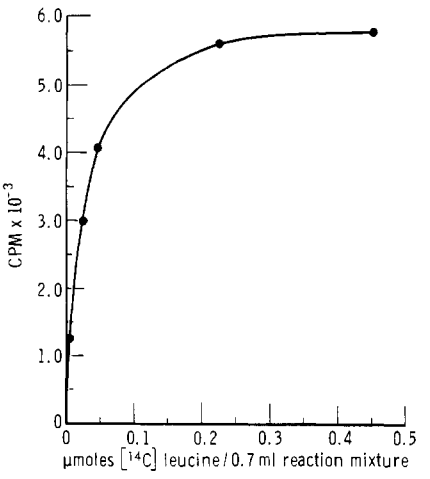

FIGURE 4: Effect of increasing concentrations of [^{14}C]-leucine on incorporation into TCA-Tung precipitable material in complete ribosomal brain cell-free system.

TABLE I: Amino Acid Mixture.

Amino Acid	μmoles/ml
Alanine	9.7
Phenylalanine	5.2
Aspartic acid	12.6
Arginine	5.3
Cysteine	1.1
Glutamine	5.5
Glutamic acid	14.8
Glycine	13.0
Histidine	1.9
Hexosamine	5.6
Isoleucine	5.8
Lysine	8.2
Methionine	1.6
Proline	12.9
Serine	17.9
Threonine	19.1
Tyrosine	3.3
Tryptophan	1.5
Valine	15.2

micromil window and an average counting efficiency of 25%. Ultraviolet absorbance of cell fractions was determined at 2600 and 2800 A.

Preparation of Cell Fractions. Microsomes, ribosomes, and pH 5 fractions were prepared from rabbit brain by methods previously described (Rubin and Stenzel, 1965), and outlined in the flow sheet in Figure 1.

Incorporating System. The complete incorporating system contained in 0.7 ml: 25 μmoles of Tris, pH 7.4 at 25°, 25 μmoles of KCl, 5 μmoles of $MgCl_2$, 10 μmoles of GSH, 0.5 μmole of ATP, 0.125 μmole of GTP, 2.5 μmoles of 2-phosphoenolpyruvic acid, 30 μg of pyruvate kinase, 0.1–0.2 mg of ribosomes, pH 5 protein, 0.5 μc of [^{14}C]leucine, and 0.03 ml of an amino acid mixture tabulated in Table I. ATP, GTP, 2-phosphoenolpyruvic acid, [^{14}C]leucine, and the amino acid mixture were neutralized to pH 7.4 with 1 N KOH and stored at −30°. Microsomes served as a source of ribosomes in some experiments.

Results

Characteristics of Cell Fractions. Instead of attempting to obtain maximum yields of microsomes or ribosomes, brains were processed as rapidly and as gently as possible so as to minimize degradation. Connective tissue was dissected away and the cerebral cortexes were re-

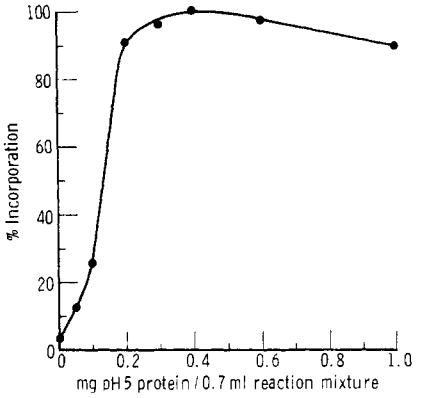

FIGURE 5: Effect of pH 5 protein on incorporation of [^{14}C]leucine in the brain cell-free system. In this experiment each 0.7-ml system contained 0.2 mg ribosomes.

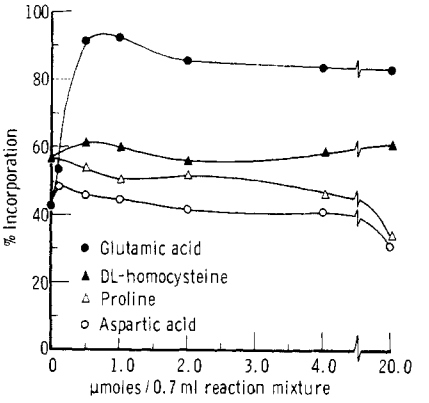

FIGURE 7: Effects of glutamic and aspartic acids, DL-homocysteine, and proline on incorporation of [^{14}C]-leucine into TCA-Tung precipitable material in brain ribosomal cell-free systems incubated without amino acid mixture.

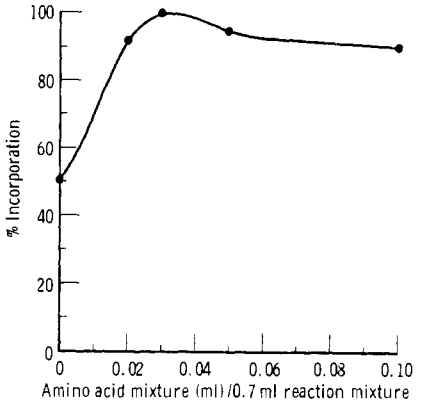

FIGURE 6: Effect of amino acid mixture (see Table I) on incorporation of [^{14}C]leucine into TCA-Tung precipitable material in brain ribosomal cell-free system.

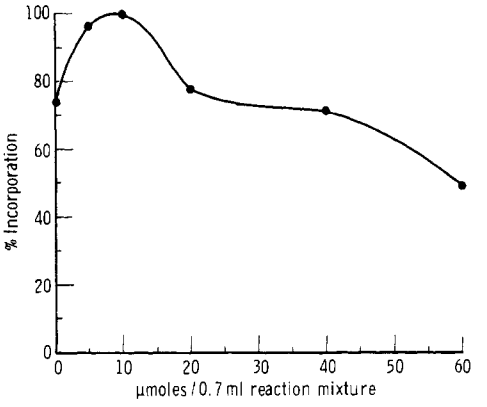

FIGURE 8: Effect of GSH on incorporation of [^{14}C]-leucine into TCA-Tung precipitable material in brain ribosomal cell-free system.

moved and homogenized by five strokes with an A handle of a Dounce tissue homogenizer, followed by one with a B handle. The interval between removal of brains and shearing of cells never exceeded 60 min. The yield of cortical tissue per rabbit brain was approximately 5 g wet weight. From this was extracted 15–20 mg of pH 5 protein, 1–2 mg of ribosomal RNA, and 2.5–3.5 mg of ribosomal or 15–20 mg of microsomal protein. The ratio of optical density at 2600 A to that at 2800 A was 1.33 for microsomes and 1.50 for ribosomes. The pH 5 fraction contained 1.7–2.5% RNA and the OD 2600/2800 ratio was less than unity (0.88–0.90). Brain pH 5 fraction did not sediment as readily as that from reticulocytes or liver cells, and even after 60 min at 15,000*g*, the supernatant fluid was opalescent. This fraction could be stored at −30° in 0.1 M Tris pH 7.4 for at least 4 weeks without losing its ability to stimulate protein synthesis in cell-free systems. Ribosomes, on the other hand, gradually lost their ability to incorporate amino acids when stored in 0.25 M sucrose at −30° (Figure 2).

Protein Synthesis by Complete System. Optimal incorporation of [^{14}C]leucine into TCA-Tung precipitable material depended upon the presence of ribosomes or microsomes, ATP and an ATP regenerating system, pH 5 enzymes, GTP, magnesium, and potassium (Table II). Amino acids were required in the ribosomal but not in the microsomal system. From 0.8 to 1.0 mμmole of [^{14}C]leucine was incorporated per mg of ribosomal RNA. Incorporation per milligram of RNA was essentially the same in microsomal or ribosomal mixtures.

Incorporation of isotope into TCA-Tung precipitable material was linear for at least the first 10 min and reached a plateau at about 30 min (Figure 3). The time course of incorporation was the same in both microsomal and ribosomal systems. The optimal amount of [^{14}C]leucine was 0.23 μmole/0.7 ml of reaction mixture

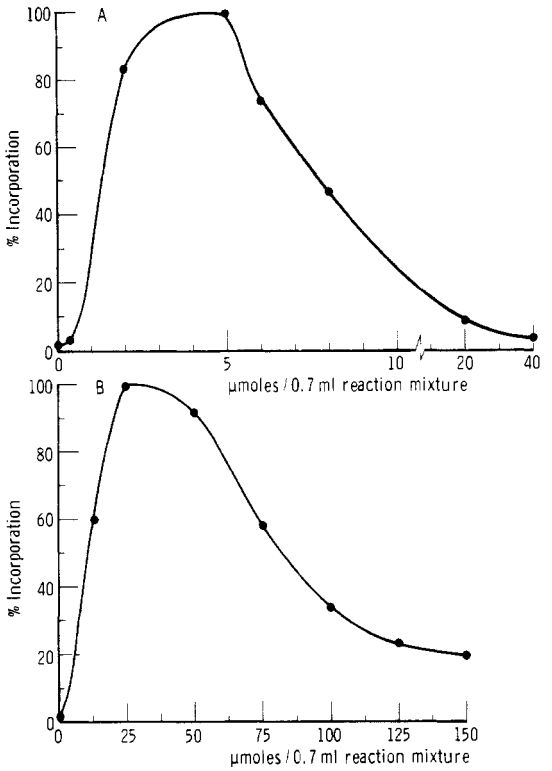

FIGURE 9: Effect of magnesium (A) and potassium (B) on incorporation of [^{14}C]leucine into TCA–Tung precipitable material in brain ribosomal cell-free system.

TABLE II: Requirements for Protein Synthesis in the Ribosomal Brain Cell-Free System.

System	% Incorporation
Complete (60 min)	100.0
Complete (0 time)	0.3
Minus ribosomes or microsomes	1.1
Minus pH 5 fraction	3.2
Minus ATP	5.0
Minus GTP	35.0
Minus 2-phosphoenol pyruvate and pyruvate kinase	50.0
Minus amino acids	53.0
Minus potassium	1.2
Minus magnesium	1.0
Minus GSH	75.0

containing 0.2 mg of ribosomes. Further increases in labeled amino acid resulted in only slight increases in incorporation (Figure 4). When cell-free mixtures, incubated for 60 min, were centrifuged at 110,000*g* for 60 min, 15% of the labeled protein remained in the supernatant fluid.

Since ribosomal systems contain less nonribosomal protein than microsomal ones and are more sensitive to alterations in the incubation mixtures, ribosomal cell-free systems were used in the following experiments unless otherwise noted.

Effect of Alterations in Ribosome and pH 5 Protein Concentrations. Incorporation was less than 1% of the complete system in the absence of ribosomes, and was a linear function of ribosome concentration up to the highest concentration tested, 280 µg of ribosomal RNA/0.7 ml of reaction mixture. In the absence of pH 5 fraction incorporation was minimal, amounting to less than 5% of the complete system. Incorporation was maximally stimulated with about twice as much pH 5 protein as ribosomes. High concentrations of pH 5 fraction resulted in slight inhibition of incorporation (Figure 5). Rabbit liver pH 5, prepared in the same way as the brain pH 5 fraction, was as effective as the brain material in stimulating amino acid incorporation.

Effect of Amino Acids. An amino acid mixture stimulated incorporation approximately twofold (Figure 6). Although not all amino acids were assayed separately, the effect of selected ones was studied. Glutamic acid alone could stimulate incorporation up to 90–95% of the complete mixture. Aspartic acid, proline, and DL-homocysteine had either no effect or depressed incorporation at high concentrations (Figure 7).

Effect of GSH. Omission of GSH from the reaction mixture resulted in a 20–30% decrease in incorporation (Figure 8). The technique of preparing GSH was found to be critical in obtaining reproducible results. Previously prepared solutions of GSH were ineffective in stimulating incorporation and gave rise to irreproducible results. It was necessary to prepare GSH immediately before use and to adjust the pH to 7.0–7.5 with 1 N KOH. Mercaptoethanol in place of GSH inhibited incorporation.

Effect of ATP, GTP, 2-Phosphoenolpyruvic Acid, and Pyruvate Kinase. Both ATP and GTP were required for maximum activity of the system. Incorporation was decreased by 95% in the absence of ATP and by 65% in the absence of GTP. Optimal levels were 0.5 µmole of ATP and 0.125 µmole of GTP/0.7 ml of reaction mixture. Either 2-phosphoenolpyruvic acid or pyruvate kinase could be omitted without effect, but when both were omitted incorporation was decreased by 50%. This observation is quite reproducible, although its mechanism remains obscure. Several possibilities are currently being investigated.

Effect of pH and Salt Concentrations. The cell-free system functioned in a narrow pH range and was optimal at pH 7.4–7.6. Raising the pH to 8.5 decreased incorporation 65%, and reducing the pH to 6.0 decreased incorporation 40%. The optimal amount of magnesium was 5 µmoles/0.7 ml (Figure 9A). The system was virtually inactive in the absence of potassium, and maximum activity was achieved at a concentration of 25 µmoles/0.7 ml (Figure 9B). High concentrations were inhibitory. Sodium in the presence of 25 µmoles of potassium resulted in inhibition of incorporation, and in the absence of potassium, sodium was ineffective

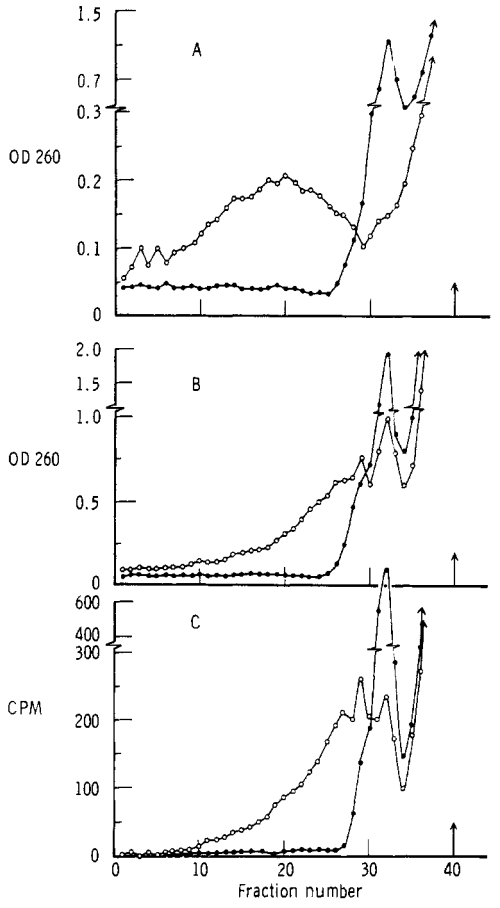

FIGURE 10: Density gradient centrifugation of brain ribosomes through 15–30% sucrose (see Methods). After centrifugation the bottoms of the tubes were punctured and fractions collected by drop counting and assayed for absorbancy and/or radioactivity. The tops of the gradients are indicated by arrows. Solid circles represent ribonuclease treated, and open circles untreated samples. A. Postmitochondrial fraction from rabbit brain, treated with 0.5% DOC. B. Ribosomes after preparation for cell-free systems (see Figure 1) and stored at −30° overnight. C. Radioactivity of fractions from the preparation illustrated in B after being incubated in the complete incorporation mixture (see Methods) containing 5 μc [^{14}C]leucine for 30 sec.

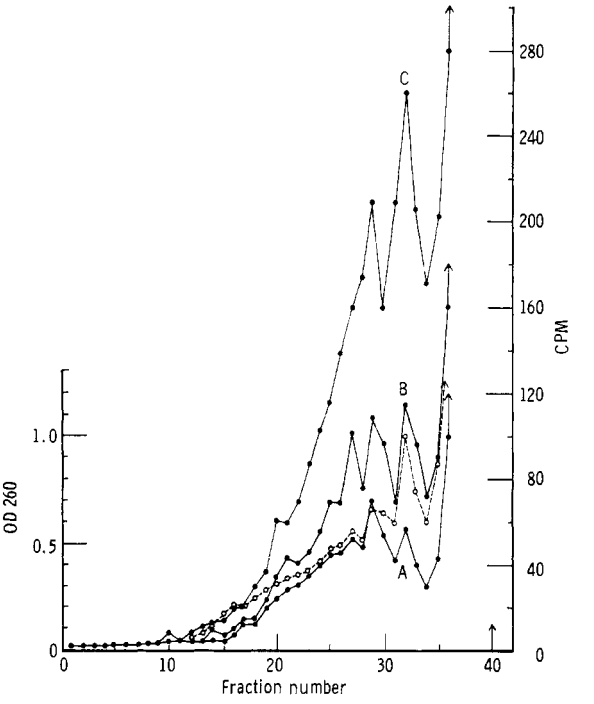

FIGURE 11: Density gradient centrifugation of brain ribosomes through 15–30% sucrose. The arrow indicates the top of the gradients. The open circles indicate absorbancy at 2600 A and the solid circles cpm. Ribosomes were prepared as in Figure 1, and incubated in the complete incorporation mixtures containing 5 μc [^{14}C]leucine for A, 30 sec, B, 45 sec, and C, 60 sec.

TABLE III: Effect of Inhibitors of Protein Synthesis on the Brain Cell-Free System.

Inhibitor	μgrams/0.7 ml	% Inhibition
Ribonuclease	1.0	97
Puromycin	50.0	88
Puromycin	100.0	93
Chloramphenicol	100.0	0
Deoxyribonuclease	20.0	0
Actinomycin D	5.0–100.0	0

in stimulating amino acid incorporation. Calcium inhibited incorporation at levels greater than 0.7–1.0 μmole/0.7 ml.

Effect of Inhibitors of Protein Synthesis and Neuropharmacologic Agents. Both puromycin and ribonuclease effectively inhibited incorporation of [^{14}C]-leucine, whereas chloramphenicol, actinomycin, and deoxyribonuclease were ineffective (Table III). A variety of agents, known to affect central nervous system function profoundly, were studied in the cell-free system. These included α-aminobutyric acid, sodium phenobarbital, strychnine, acetylcholine, hydrocortisone, lysergic acid, β,β-iminodipropionitrile, and DL-homocysteine. None had a significant effect on the incorporation of [^{14}C]leucine into TCA–Tung precipitable material.

Site of Protein Synthesis in the Cell-Free System. Sucrose density gradient centrifugation of incorporation mixtures revealed the presence of polydispersed material absorbing at 2600 A, indicating the presence of single and aggregated ribosomes (Figure 10B). The pattern is different from that of postmitochondrial brain fractions treated with DOC and immediately

centrifuged through similar gradients (Figure 10A). Under these conditions, there is a greater proportion of aggregated ribosomes and very few single ones. Treatment with 1 μg of ribonuclease at 4° for 10 min results in complete loss of heavier aggregates and the shift of all 2600 A absorbing material to the single ribosome peak. As protein synthesis proceeds, there is a gradual breakdown of polysomes. Even at 30 min, however, when the rate of [^{14}C]leucine incorporation is negligible, significant amounts of aggregated ribosomes persist. A 30-sec pulse of incorporation mixtures with [^{14}C]leucine revealed the nascent protein to be associated with both aggregated and single ribosomes, although the former had a greater specific activity (cpm per unit OD) (Figure 10C). With longer labeling periods, a progressively greater amount of nascent protein was associated with the single ribosome peak (Figure 11). Addition of ribonuclease to labeled incubation mixtures again resulted in the breakdown of polysomes, and the nascent protein remained in association with the single ribosomes.

Discussion

Our results, which are in general agreement with those of Satake *et al.* (1964), Murthy and Rappaport (1965), Suzuki *et al.* (1964), and Zomzely *et al.* (1964), indicate that brain ribosomal cell-free systems are similar to other mammalian systems in many respects. The ATP, GTP, potassium, magnesium, and GSH requirements are virtually identical with those of reticulocyte (Allen and Schweet, 1962) and liver (Sachs, 1957) systems. Relative dependence of incorporation upon amino acids has previously been reported in brain incorporating systems (Murthy and Rappaport, 1965) as well as in other cell-free systems. While the marked stimulatory effect of glutamic acid alone is interesting, it may simply reflect the preferential loss of optimal amounts of this amino acid during preparation. Limited breakdown of polysomes occurs during preparation of ribosomes for incorporation studies. Since significant amounts of single ribosomes became labeled in the cell-free system after a 30-sec pulse with [^{14}C]-leucine, it is likely that significant amounts of messenger ribonucleic acid (m-RNA) remain attached to the single ribosomes. The aggregates behave like typical polysomes and break down completely to monomers and dimers upon treatment with ribonuclease. These particles are inactive in protein synthesis.

We were unable to detect alterations in protein synthesis in the brain cell-free system in the presence of several neuropharmacologic agents. If these agents do have an effect on protein synthesis, it is thus probably not as the level of messenger readout on ribosomes. The possibility remains that drugs influencing the central nervous system effect protein synthesis at other levels. This possibility as well as the effect of various states of excitation is now being investigated and will be the subject of subsequent reports.

The brain cell-free system is composed of ribosomes from a variety of cell types, including various neuronal and glial elements. There are undoubtedly complex relationships among the metabolic activities of these various cells and alterations arising from excitation may not be reflected in cell-free systems derived from the whole. However, methods are now becoming available for the separation of neurons from glia, and these isolated cells, when derived from specific brain areas, may reveal variations in protein synthesis obscured by the noise of present brain cell-free systems.

The same considerations apply to the identification of the products of protein synthesis in this system. We have identified (Rubin and Stenzel, 1965) a tissue-specific protein as one end product of the brain *in vitro* system, but the nature of the study precluded determination of its neuronal or glial origin. Studies utilizing components of separated cells from specific brain areas will be necessary in order to determine relationships between electrical activity and cellular metabolism.

Acknowledgments

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