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Protein Synthesis Systems from Rat Brain*

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ABSTRACT: Two distinct cell-free protein synthetic systems have been isolated from brain tissue of 18–21 day old white rats, one based on purified ribosomes and the other on mitochondria. The ribosomal system was typical in its requirements for pH 5 enzymes and an exogenous source of adenosine triphosphate (ATP), and its almost complete inhibition by ribonuclease (RNAase).

The mitochondrial system was one of the most active yet reported. It had no requirement for pH 5 enzymes or exogenous ATP, was not inhibited by RNAase, but was moderately inhibited by 0.5 μmole of dinitrophenol, 1.6 mμmoles of rotenone, or 2 μg of antimycin-A per ml. The activity was sensitive to the concentrations of inorganic phosphate, adenosine diphosphate (ADP), and ATP in the medium. Various neurochemicals had specific effects, notably stimulation by γ-aminobutyric acid in both systems. Proteins from the ribosomal system were fractionated on DEAE-cellulose columns, yielding about 3.5% of the label in a soluble acidic protein fraction. Most of the label, however, was in a protein fraction associated with ribosomal ribonucleic acid (RNA). In presence of an artificial messenger, polyuridylic acid, the system incorporated [^{14}C]-phenylalanine at several times the standard rate, provided the ribosomes were first incubated in a KCl medium low in ATP. Relations of these protein synthetic systems to functional activity of neurons are considered, including a possible role of mitochondria in synthesis of synaptic vesicle proteins.

Two distinct cell-free protein synthetic systems have been isolated from immature rat brain, one localized in purified ribosomes and one in mitochondria. These systems, both highly active, differ in various biochemical properties. The purpose of this paper is to describe and contrast the properties of these two systems, as a contribution to an eventual understanding of functional aspects of protein synthesis in brain.

Ribosomal System

There have been several recent studies of ribosomal protein synthetic systems from brain, including those of Zomzely *et al.* (1964) on rat, of Rubin and Stenzel (1965) on rabbit, and of Murthy and Rappoport (1965a) on rat. A cell-free microsomal system from guinea pig brain has been characterized by Satake *et al.* (1964).

Experimental Procedures

We used immature rats in view of reports (Murthy and Rappoport, 1965b) that immature rat brains provided more active enzyme extracts. The rats were male, Sprague–Dawley strain (Simonsen Laboratories, White Bear Lake, Minn.), 18–21 days old, approximately 45 g. The preparation of ribosomes followed that devised by Munro *et al.* (1964) for the liver system, with a fractionation procedure as outlined in Figure 1. Electron micrographs of the ribosomal preparations, for example, Figure 2, show a fairly high proportion of double ribosomes as well as some larger aggregates. Ultracentrifuge data on this point will be discussed later.

The standard ribosomal system is summarized in Table I. Ribosomes (0.6–0.7 mg of protein) and pH 5 enzymes (1.0–1.5 mg of protein) were separately suspended in medium M. The ATP[1] and GTP, pH 5 enzymes, and ribosomes were added in that order to medium M in 13 mm × 10 cm tubes held in a constant temperature block, and the run was started by addition of labeled [^{14}C]leucine. The reaction was stopped after

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[1] Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate; GABA, γ-aminobutyric acid; ACH, acetylcholine; EPI, epinephrine; NEPI, norepinephrine; DOPA, *o*-dihydroxyphenylalanine; CPZ, chloropromazine; SERO, serotonin; α-KGA, α-ketoglutarate; DPN, 2,4-dinitrophenol; GDP, guanosine diphosphate; IDP, inosine diphosphate.

TABLE I: Standard Ribosomal System.[a]

0.25 M Sucrose	Medium M
0.02 M Tris pH 7.6	
10 μmoles of $MgAc_2$	
40 μmoles of NaCl	
100 μmoles of KCl	
6 μmoles of mercaptoethanol	
5 μmoles of ATP	
0.1 μmole of GTP	
1 mg of pH 5 enzyme protein	
0.7 mg of ribosomal protein	
0.5 μc [^{14}C]L-leucine	

[a] Standard volume was 1.0 ml. Runs were made at 37°. Proteins were assayed by method of Lowry *et al.* (1951).

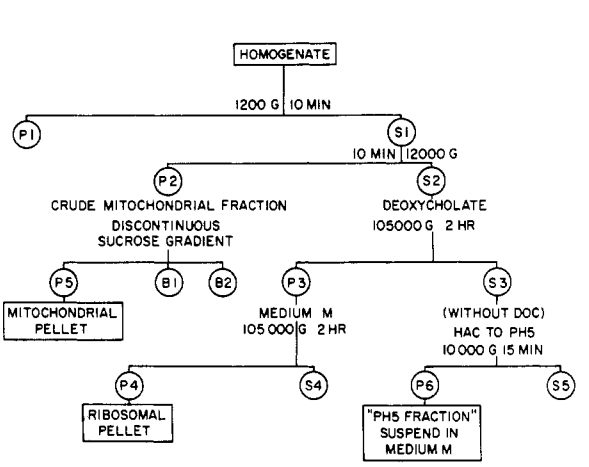

FIGURE 1: Fractionation of rat brain tissue.

addition of excess unlabeled amino acids and protein precipitated by 2 ml of 5% trichloroacetic acid.

The precipitates were prepared for counting so as to remove any unincorporated radioactivity and the bulk of ribonucleic acid (RNA) and lipids. The initial trichloroacetic acid precipitate was dissolved in 0.25 M NaOH, 0.02 mM in unlabeled amino acid, and reprecipitated with 5% trichloroacetic acid. The second precipitate was washed twice with 5% trichloroacetic acid. In the course of the trichloroacetic acid washing, the suspensions were heated at 90° for 15 min to solubilize any [^{14}C]aminoacyl transfer RNA. Lipids were extracted by washing twice with ethanol–ether solution, 3:1 by volume. The precipitates were freed of solvent by heating the tubes in a hot water bath, dissolved in concentrated formic acid, plated onto weighed aluminum planchets, and dried before counting in a thin-window automatic gas flow counter. Self-absorption was practically constant. Results were corrected for background and zero-time counts (both of which were small) and recorded as counts per minute per milligram of total protein. The protein was calculated from the mass of dried precipitate and protein analyses of the ribosomal and pH 5 fractions (Lowry *et al.*, 1951). From the stated specific activity of the amino acid and calibration of the counter against standard mixtures of protein and labeled amino acid, these figures were converted to $\mu\mu$mole of incorporated amino acid per milligram of ribosomal protein. The specific activities of the leucine (Schwarz Bioresearch Co., Orangeburg, N. Y.) varied from 131 to 240 μc/μmole. The standard time of incubation was 60 min, the total incorporation rising to a plateau at 40–60 min.

Amino Acid Incorporating Conditions. The results of standard tests under various conditions are summarized in Table II. The requirement of the system for phosphorylating agents of high free energy in the form of ATP and GTP was apparent. At the magnesium, ATP, and pH 5 enzyme levels used in the complete system, there did not appear to be a requirement for unlabeled amino acids, but the pH 5 precipitate was essential.

TABLE II: Characterization of Requirements of Rat Brain Ribosomal Amino Acid Incorporating System.[a]

Conditions	Ribosomal Protein (cpm/mg)	Leucine Incorpd ($\mu\mu$moles)
Complete system	9650 ± 300	240
− pH 5 fraction	120 ± 5	2.9
− ATP	300 ± 100	7.4
− GTP	4360	115
ATP 0.5 μmole + ATP generating system	6200 ± 2800	155
Complete system	9850 ± 250	375
+ Unlabeled amino acids	8710 ± 450	332

[a] Standard incubation conditions were given in Table I. Incubation time was 1.0 hr. ATP generating system was 10 μmoles of phosphoenolpyruvate +7.5 μg of pyruvate kinase. The mixed unlabeled amino acids were 19 different amino acids at concentrations of 10 mM each. Means of two runs each are given for two different preparations.

The system required both a bivalent and a univalent cation for optimal incorporating activity. The optimal Mg^{2+} concentration was 10 mM. Mn^{2+} and Ca^{2+} were strongly inhibitory when added to replace half the Mg^{2+} requirement and even more so in larger amounts. The activity of the system was maintained when K^+ was replaced by Na^+ or NH_4^+ as long as the total ionic concentration was kept constant. There was no stimulation by NH_4^+. Florini (1964) reported stimulation by NH_4^+ in a protein synthesis system derived from rat muscle. Na^+ was not inhibitory even when it completely

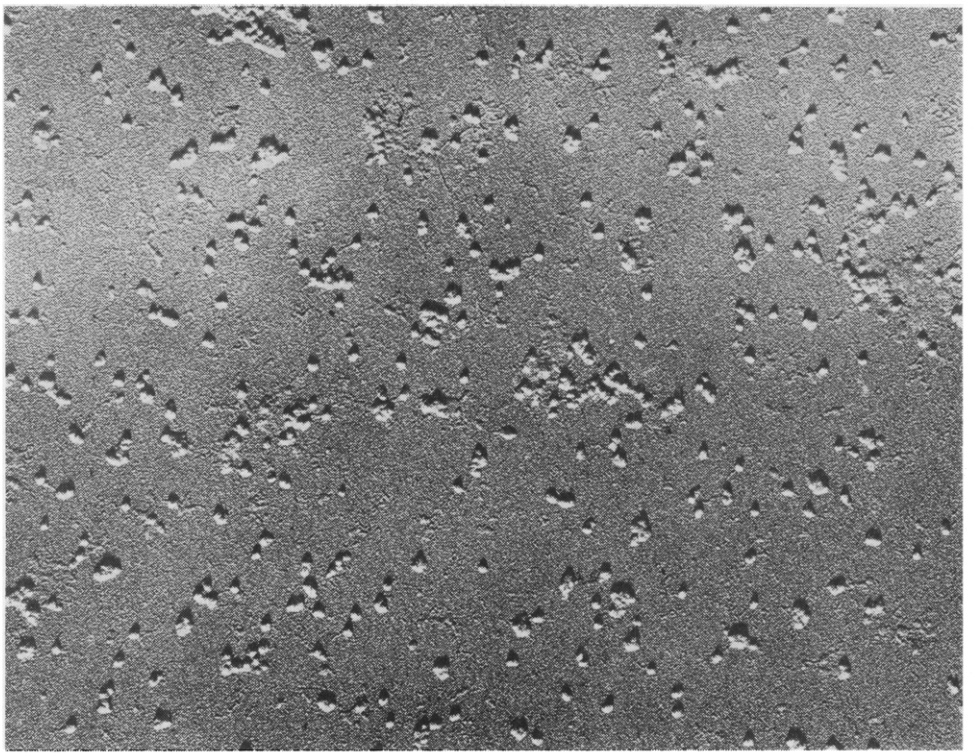

FIGURE 2: Ribosomes from rat brain, shadow-cast with platinum (80,000×).

replaced K^+. Rb^+ and Cs^+ also functioned as satisfactory replacements for K^+.

Effects of possible inhibitors of various stages of protein synthesis are summarized in Table III. Incorporation of labeled amino acids was strongly inhibited by puromycin and RNAase, and slightly inhibited by cycloheximide. It was resistant to chloramphenicol, deoxyribonuclease (DNAase), and actinomycin-D. In these respects our system was fairly typical of ribosomal systems of mammalian origin (Hultin, 1964).

TABLE III: Response of Ribosomal Amino Acid Incorporating System to Inhibitors.[a]

Conditions	Ribosomal Protein (cpm/mg)	Leucine Incorpd ($\mu\mu$-moles)
Complete system	9850 ± 250	375
+5 μg of puromycin	1940 ± 250	74
+5 μg of RNAase	185 ± 2	5
+25 μg of chloramphenicol	13500 ± 1100	510
Complete system	7485 ± 925	285
+5 μg of DNAase	7590	290
+5 μg of actinomycin-D	7850 ± 800	323
Complete system	12770 ± 810	157
+200 μg of cycloheximide	9700 ± 700	118

[a] Means of two runs each are given in three different preparations.

Mechanism of Amino Acid Incorporation. The mass distribution of ribosomes and polysomes was investigated by means of a Beckman Model E ultracentrifuge using Schlieren optics. The ribosomes were suspended in medium M and centrifuged at 21,740 rpm. Ribosomes incubated for 1 hr under conditions of protein synthesis had $s_{20,w}$ values (uncorrected for concentration) of 60, 87, 106, and 132, the peaks due to each of the two lighter fractions being about 10× higher than those due to the heavier fractions. Ribosomes which had not been incubated had $s_{20,w}$ values (uncorrected for concentration) of 55, 87, 123, and 129. In the reticulocyte system, Warner *et al.* (1963) identified triple ribosomes at 134 S and double ribosomes at 108 S. The ultracentrifuge data indicate that an appreciable proportion (~10%) of double and triple ribosomes are present in our system, and suggest some cleavage of triples to doubles under the incubation conditions. Electron microscopic examinations showed the preparations to be less aggregated following incubation. When the Mg^{2+} concentration of the medium was reduced from 10^{-2} M to 10^{-5} M, only subunits at 42 S and 25 S could be detected by ultracentrifugal analysis.

The relative incorporation of labeled leucine in hot trichloroacetic acid insoluble material bound to ribosomal aggregates of various sizes was determined in the following manner. Ribosomes were reisolated from a standard incubation mixture by centrifugation for 2.0 hr at 30,000 rpm in the 30 rotor of a Spinco Model L

ultracentrifuge, then resuspended in 2 ml of medium M. This suspension was layered onto a linear 23-ml gradient of 0.3 M–1.0 M sucrose in medium M and centrifuged for 1.0 hr at 24,000 rpm in the 25.1 rotor. The ribosomal material was determined in 0.8-ml fractions by absorption at 260 mμ. The material was then precipitated with trichloroacetic acid, washed, and plated as usual. The highest activities coincided with peaks just above the middle of the gradient, having S values of about 80 and above (Figure 3). Thus single 80S ribosomes as well as polysomes of two, three, or more units carried nascent polypeptide chains.

Response to Drugs. We investigated the response of the ribosomal system to several substances involved in nervous transmission and others such as tranquilizers which affect the nervous system in some specific way. The results are summarized in Figure 4. In experiments with preparations of high activity a marked stimulatory effect of GABA was observed. Average maximal stimulation amounted to 60% and was obtained at a concentration of 20 mM.

The brain system was compared with a ribosomal system obtained from the livers of the same rats, essentially the same as that described by Munro *et al.* (1964). Under standard test conditions the activity of the brain system was about five times higher than that of the liver system. The difference in activities was found to originate in the pH 5 fractions. In each system, the amount of ribosomal protein was kept constant and the amount of pH 5 precipitate varied. Both were incubated under the same conditions, since these were optimal for both. The greater activity of the brain pH 5 fraction was probably not due simply to a higher concentration of activating enzymes, since addition of more liver pH 5 fraction did not restore activity to that achieved with the brain pH 5 preparation. The different activities of brain and liver systems may be due to an organ specificity in the code, or to some control mechanism present in liver but not in brain (Hoagland and Askonas, 1963).

In the liver system, only a slight stimulation of amino acid incorporation by GABA was ever noted. Dopamine, epinephrine, norepinephrine, and acetylcholine had no marked effects in either system.

Serotonin had a marked and reproducible inhibitory effect on the brain system but no appreciable influence on the liver system. Chlorpromazine reproducibly inhibited the brain system at a level at which there was no inhibition in the liver system, or in a cell-free system from mouse brain (Shuster and Hannam, 1964).

Response to Artificial Messengers. The incorporation of [^{14}C]phenylalanine into protein by the brain system was stimulated by addition of polyuridylic acid (poly-U), with maximal response at levels of 100 μg/ml. The standard ribosomal fraction showed the greatest stimulation by added poly-U, more than crude microsomes, polysomes, or ribosomes sedimented from the post-microsomal supernatant. It was not possible to enhance the effect of poly-U by pretreatment of the ribosomes, such as preincubation or exposure to low Mg^{2+} before they were added to the incubation mixture: such pretreated ribosomes usually showed less response to

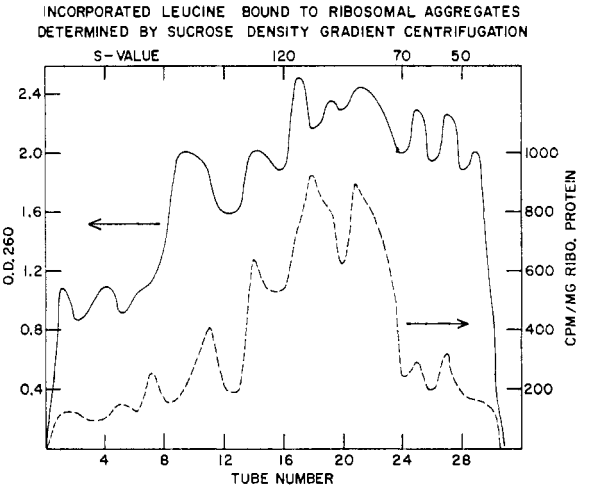

FIGURE 3: [^{14}C]Leucine incorporated in polypeptide chains and bound to ribosomes and polysomes as determined by sucrose density gradient fractionation of the ribosomal system after a standard incubation.

artificial messenger than did those simply added to the system without pretreatment.

Ribosomes were preincubated in 0.5 M KCl under conditions of protein synthesis and reisolated as described by Arlinghaus *et al.* (1964). The effect of added poly-U was greatest in those cases in which the standard activity of the system was below the maximum, which was about 90 μμmoles of [^{14}C]phenylalanine/mg of ribosomal protein/hr in these systems. Addition of 100 μg of poly-U would raise this figure to about 120. If, however, the activity without poly-U was lower, *e.g.*, 25, addition of 100 μg of poly-U would raise it to about 100. The results suggest competition between added synthetic messenger and endogenous messengers. When this KCl incubation was carried out with half the standard concentrations of ATP and GTP, a very marked stimulation of poly-U, up to eightfold, was obtained, but this was relative to a much lowered activity in the absence of poly-U (~13). This result indicated that ribosomes rolled off endogenous messenger in the course of preincubation and could then attach themselves to added synthetic messenger.

No ambiguity of the code was observed with poly-U and leucine; *i.e.*, there was no enhanced incorporation of [^{14}C]leucine in the presence of poly-U, even at high concentrations of Mg^{2+}.

Protein Separations. Proteins formed by the brain ribosomal system were separated by methods similar to those of Rubin and Stenzel (1965). The soluble fraction consisted of proteins of basic, neutral, and acidic character. There were definite peaks of high activity in the region of slightly acidic and acidic proteins. About 4% of the total radioactivity incorporated appeared in these soluble proteins. They do not appear to be restricted to such narrow bands of eluant as were the soluble proteins obtained from rabbit ribosomes (Rubin and Sten-

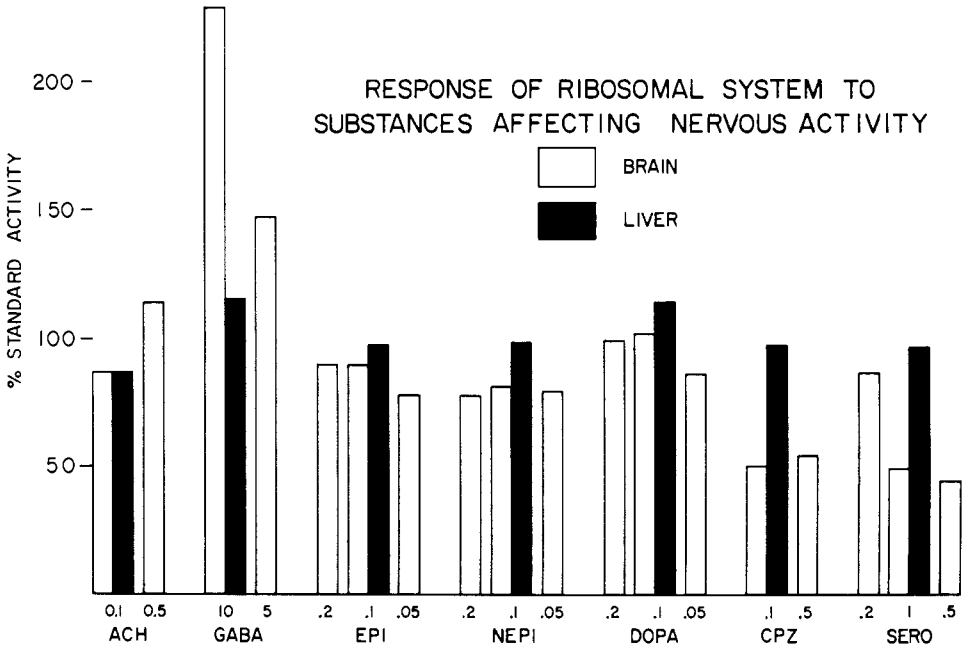

FIGURE 4: Response of ribosomal systems to substances affecting nervous activity. Tests were made at various millimolar concentrations.

zel, 1965). A second protein fraction was obtained by solubilizing the ribosomal pellet by exposure to low (Mg^{2+}). This contained about 3.5% of the total radioactivity.

The structural protein of the ribosomes was solubilized by treatment with cold glacial acetic acid in a Teflon-glass homogenizer for 45 min at 4° (Waller and Harris, 1964). Under these conditions, about 10% of the ribosomal protein remained associated with RNA and could not be separated by further treatment with cold acetic acid. The structural protein fraction of the ribosomes was not labeled, except for a slight label in a highly acid protein that was probably not structural, but a carryover from the soluble protein fraction

The label found in various fractions is summarized in Table IV. It is evident that the largest part of the label remained tightly associated with ribosomal RNA. This protein may include partially completed peptide chains as well as completed proteins insoluble in cold glacial acetic acid.

TABLE IV: Fractionation of Proteins Labeled with [^{14}C]Leucine in Standard Ribosomal Protein Synthetic System.

Fraction	% Total Protein	% Total Count	Relative Specific Activity
Soluble	32.2	3.5	110
Low [Mg^{2+}]	32.9	3.4	100
Ribosomal	24.7	0.3	1
Residual	10.2	92.8	9100

Mitochondrial System

There have been detailed reports on protein synthesis in mitochondria from liver (Roodyn *et al.*, 1962) and heart (Kroon, 1963), and recently by Klee and Sokoloff from brain (1965). The brain mitochondrial system is of

TABLE V: Comparison of Two Protein Synthetic Systems from Rat Brain.

Ribosomal	Mitochondrial
1. Absolute requirement for pH 5 enzyme fraction.	1. No requirement for pH 5 enzyme fraction.
2. No substrate for oxidative phosphorylation needed.	2. Activity enhanced by α-ketoglutarate.
3. Requires exogenous source of ATP.	3. Does not require exogenous source of ATP.
4. Stable on storage at 0° for 1 week.	4. Dead within hours without α-KGA.
5. Inhibited 50% by 0.5 mM SERO	5. Stimulated 90% by 0.5 mM SERO
6. Inhibited 98% by RNAase.	6. Not inhibited by RNAase.

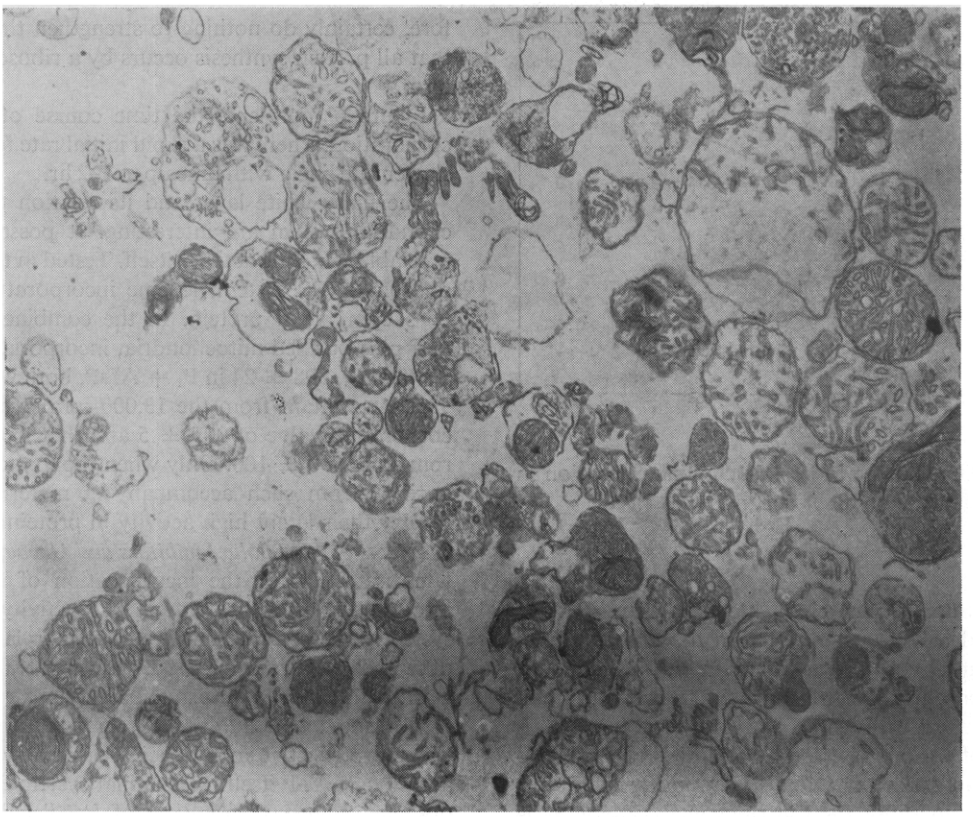

FIGURE 5: Mitochondria from rat brain, fixed in buffered osmium tetroxide, embedded in Araldite, sectioned with a Porter-Blum MT-2 microtome, stained with Reynolds lead citrate (40,000×).

special interest because of the high concentration of mitochondria in the synaptic regions of neurons.

Our results have shown that brain mitochondria can furnish an unusually active protein synthesizing system. There is little doubt that this mitochondrial protein synthesis system is qualitatively different from the ribosomal system. Table V summarizes the contrasts between the mitochondrial and ribosomal systems, which will be detailed in the subsequent discussion.

Experimental Procedures

Brain is a difficult organ from which to obtain reasonably uncontaminated mitochondria. A preparation described by Lovtrup and Zelander (1962) appeared to yield suitable mitochondria and we therefore adapted it to our work.

The rats were of the same kind as used for the ribosomal system. Excised brains were kept in crushed ice. After removal of white matter and blood vessels, brains were washed with 0.44 M sucrose and transferred to a chilled-glass tissue homogenizer (A. H. Thomas); 10% wet weight of tissue in medium was used. Homogenization was carried out for 1.0–1.5 min in a cold room. The homogenate was centrifuged for 10 min in a Spinco L centrifuge (rotor SW-25.1) at 5000 rpm. The supernatant was decanted and centrifuged at 13,000 rpm for 15 min. The pellet was washed with 20 ml of 0.44 M sucrose and centrifuged at 9000 rpm for 15 min. A fluffy white layer on top of the mitochondrial pellet was removed. This contained broken mitochondria, microsomal and myelin fragments, and possibly specific inhibitory factors, since when it was retained with the mitochondria, the protein synthetic activity of the system was reduced by almost an order of magnitude. The washing operation was repeated twice. The last precipitate was resuspended in 0.44 M sucrose for subsequent tests.

An electron micrograph of a typical preparation is shown in Figure 5. Electron microscopic examination after incubation in the protein synthesizing system showed that the mitochondria remained intact and essentially unaltered in appearance.

Amino Acid Incorporating Conditions. It is important to keep in mind that mitochondrial protein synthesis *in vivo* may be inhibited by factors similar to those which drastically lower the activity of the *in vitro* system. The system of highest activity is not necessarily the best example of the functional system in neurons.

There is evidence that mitochondria contain all the citric acid cycle enzymes and that oxidation of glucose in the brain proceeds mainly *via* pyruvate through the citric acid cycle (Gallagher *et al.*, 1956). It has been reported that brain mitochondria are distinguished from other mitochondria by their possession of glycolytic enzymes (Balazas and Richter, 1960). Considering these

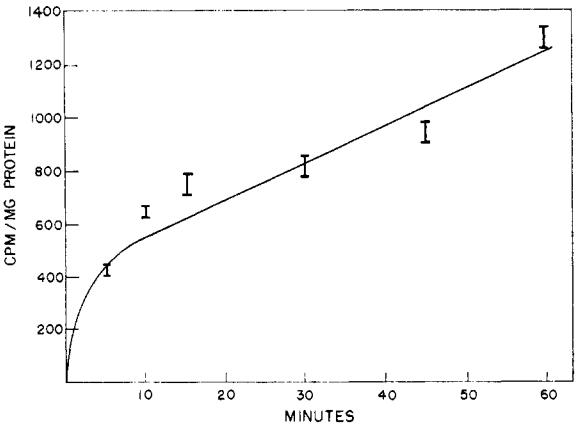

FIGURE 6: Time course of [^{14}C]leucine incorporation in mitochondrial system (described in text).

facts, pyruvate, succinate, malate, fumarate, GABA, glucose, and α-KGA were tested in the mitochondrial system. Highest activity was found in α-KGA, with an optimal concentration of 5 mM. The pH optimum of the system was between 7.2 and 7.6.

Results of various tests on several different preparations are summarized in Table VI. Counting conditions were similar to those for the ribosomal system. It is significant than the system was stimulated by RNAase, presumably by an effect external to the mitochondria, rather than after penetration into the mitochondria.

Our present preparation is among the most active mitochondrial materials yet reported, its optimal activity being about four times that of the preparation from heart mitochondria described by Kroon (1963). If the observed protein synthesis is due to ribosomes, their activity within the intact mitochondria (based on RNA content) must be at least 100 times greater than that in purified ribosome preparations. In view of Kroon's data on fragmented mitochondria such an interpretation would require that ribonuclease activity be effectively inhibited in mitochondria. Our data, therefore, certainly do nothing to strengthen the hypothesis that all protein synthesis occurs by a ribosomal mechanism.

Figure 6 represents the time course of leucine incorporation. There was a rapid initial rate followed by a slower rate linear with time up to 1–2 hr.

The fluffy white layer and its relation to the mitochondrial system are interesting. It possesses an appreciable synthetic activity itself. Tested in the inorganic phosphate (P_i) system, leucine incorporation was 393 ± 5 cpm/mg of protein. In the combined system of fluff plus purified mitochondria, incorporation was 480 ± 48 in P_i, 398 ± 24 in P_i + ADP, both with α-KGA. The supernatant from the 13,000-rpm spin had an incorporation value of 305 ± 5 and that from the 9000-rpm spin, 530 ± 100. Only when the mitochondria are purified from such accompanying materials do they display their latent high activity in protein synthesis.

Effects of Metabolic Inhibitors and Uncouplers. In isolated mitochondria the incorporation of amino acids into protein is dependent on the activity of enzyme systems involved in oxidative phosphorylation, as was observed by Reis *et al.* (1959) and by Kalf (1963). In work with rat liver mitochondria, Truman and Korner (1962) noted that addition of an oxidizable substrate was more effective in enhancing protein synthesis than addition of ATP itself. In a similar system Bronk (1963) observed that incorporation of [^{14}C]leucine was not sensitive to inhibitors of ATP formation, *e.g.*, it was not inhibited by oligomycin which blocks the terminal phosphorylation step that yields ATP (Kulka and Cooper, 1962). On the other hand, he observed that incorporation was blocked by antimycin-A or DNP, the former reagent interrupting the respiratory chain between cytochromes *b* and *c*, and the latter uncoupling phosphorylation from respiration but also inhibiting some nonphosphorylative oxidation processes (Racker, 1965). Kroon (1964) found about 75% inhibition by 0.5 mM DNP in both rat liver and beef heart mitochondria. Thus there is a definite possibility that protein synthesis in mitochondria is driven by a high energy intermediate of oxidative phosphorylation that itself

TABLE VI: Incorporation of [^{14}C]Leucine by Mitochondria (B).[a]

Experimental Conditions	ADP–P_i Medium		P_i Medium	
	cpm/mg of Protein	μμmoles of Leucine	cpm/mg of Protein	μμmoles of Leucine
No substrate	1201 ± 22	6.7	1271 ± 72	16.9
+5 mM α-KGA	1375 ± 145	7.7	1379 ± 272	18.1
+5 mM α-KGA + 2 μg of RNAase	1642 ± 65	9.2	1947 ± 356	26.0
+5 mM succinate	1032 ± 149	5.8	872 ± 86	11.5

[a] Incubation mixture consisted of medium M + 2 μmoles of ADP, 10 μmoles of K_2HPO_4, and 1 μmole of GTP. This is referred to as an ADP–P_i system. About 2 mg of mitochondrial protein was present in each tube and 0.5 μc of [^{14}C]L-leucine. pH 5 protein when present was 1.0–1.5 mg. Total volume was 1.0 ml, pH 7.6. Mitochondria were suspended in 0.32 M sucrose, 20 mM Tris. Incubation period was 60 min at 37° with air as the gas phase.

may or may not contain phosphate. Since the work of Bronk (1963), in particular, referred to systems of extremely low protein synthetic activity (about 50 cpm/mg of protein/hr), it was of interest to examine similar effects in our much more active brain mitochondrial systems.

Such studies were complicated by the effects of ADP and inorganic phosphate (P_i) levels on the respiratory cycle and by implication on protein synthesis in mitochondria. Chance and Hollunger (1961) have emphasized concentration of ADP as the most critical determinant of respiration rate in both intact cells and isolated mitochondria. Rate of respiration probably depends on the concentrations of both reactants, ADP and P_i. P_i is required for coupled respiration and it seems likely that the active swelling of mitochondria, which is stimulated by P_i, is related to some step involving phosphate uptake in oxidative phosphorylation (Raaflaub, 1953; Packer, 1961). Lehninger (1962) has shown that mitochondria are most susceptible to swelling during respiration in absence of ADP and ATP. Likewise Brierley *et al.* (1962) found that phosphate accumulation in the presence of Mg^{2+} is diminished when ADP is present as phosphate acceptor. A possible effect of the medium on the transport properties of the mitochondrial membrane is another factor that must be considered in studying protein synthesis in intact mitochondria.

In the light of this background it seemed necessary to carry out experiments in both the presence and absence of ADP. One medium (P_i) was 10mM in K_2HPO_4, and the other (ADP–P_i) was 10 mM in K_2HPO_4 and 2 mM in ADP.

As shown in Table VI the addition of ADP at a concentration of 2 mM consistently inhibited the incorporation of leucine, indicating that perhaps formation of free, extramitochondrial ATP and protein synthesis were competitive processes involving the same high energy intermediate(s).

TABLE VII: Response of Mitochondrial Amino Acid Incorporating System (B) to Possible Inhibitors.[a]

Experimental Conditions	% Standard Activity		
	Medium		
	ADP–P_i	P_i	ATP
Standard	100	100	100
+ 3 μg of oligomycin	124	101	...
0.5 μmoles DNP	69	106	35
5 μmoles Na azide	29	46	68
1 μmole cyanide	38	79	29
2 μg antimycin-A	64	89	92
0.5 μmole amytal	102	150	78
+ 2 μg of RNAase	100	137	...

[a] The assay systems used were those described in Table VI, except for those in the column labeled ATP: here 5 μmoles of ATP, but no ADP or P_i was added. Standard activities were: for ADP–P_i 12.0 μμmoles, for P_i 14.0 μμmoles, and for ATP 14.2 μμmoles of leucine incorporated)/mg of protein/hr.

The results of tests with various respiratory inhibitors and uncoupling agents are summarized in Table VII. The figures are cited as per cent standard activity. They are the means of at least two runs and in some cases include two sets of duplicates on different mitochondrial preparations.

The classical respiratory (cytochrome oxidase) inhibitors cyanide and azide produced definite inhibition under all conditions tested, but amytal and antimycin-A, which are believed to interfere with electron transport in the region of the first and the second phosphorylation site, were without any pronounced effect. Similarly, oligomycin, an inhibitor of coupled (*i.e.*, phosphorylation-linked) respiration, did not produce any inhibition. DNP, the most commonly used uncoupler, appeared to produce ambiguous results: no inhibition was observed in the P_i medium, a moderate inhibition in the ADP–P_i medium, and very significant inhibition in a medium containing ATP but no added ADP and P_i.

It appeared worthwhile to explore further the possible relationships between oxidative phosphorylation and protein synthesis in these highly active mitochondrial preparations. First we changed our assay conditions to conform more closely to those generally employed in studies on respiratory phosphorylation. For this purpose we suspended and incubated our mitochondria in the following medium (ME) instead of our standard medium M: 0.3 M mannitol, 0.02 M EDTA, 10 mmoles of Mg^{2+}, 20 mmoles of K^+, 10 mmoles of Tris pH 7.4, and 10 mmoles of P_i, and usually 5 mmoles of α-KGA as substrate. This substitution increased the specific activity from 1450 ± 44 counts (19 μμmoles) to 2312 ± 100 counts (31 μμmoles)/min per mg of mitochondrial protein. On the other hand isolation of mitochondria in a 0.3 M mannitol–0.1 M EDTA medium, a procedure claimed by D. Ozawa, K. Sera, H. Takeda, and H. Handa (personal communication, 1965) to yield rat brain mitochondria with high respiratory control, produced particles with somewhat diminished incorporating ability, which in electron micrographs appeared to be more contaminated by other cellular constituents. Since ME medium contains an ionic complement quite different from that of M medium, we had to establish that these changes did not materially affect incorporating activity: increasing the Mg^{2+} concentration to 40 mM, omitting K^+, or increasing it to 100 mM at most affected activity by ±10%.

Both medium M and medium ME contain 0.1 mM GTP, added originally because of: (a) the well-established requirement for this component in ribosomal protein synthesis. However, in the present instance GTP might fulfill three additional functions; (b) it might provide an alternative source of high-energy intermediates and thus obscure the effects of respiratory inhibitors and uncouplers; (c) after transphosphorylation or hydrolysis to GDP it could be used as an essential factor in the substrate-level, DNP-independent phosphorylation linked to the oxidation of α-KGA, a function for which IDP should be equally effective, and (d) it might simi-

larly function as primary or secondary phosphate acceptor in some other essential step in the over-all sequences. In order to dissociate these three possible effects of GTP, we investigated: (a) leucine incorporation in the complete absence of GTP and the effect of inhibitors thereon; (b) substitution of equal concentrations of other nucleoside triphosphates in place of GTP, and (c) substitution of other nucleoside diphosphates for GDP and effects of lowering the GDP concentration from 0.1 mM downward. The results of these experiments are shown in Table VIII. It will be seen that with GTP omitted, antimycin-A and rotenone (a particularly effective respiratory inhibitor at the first phosphorylation site) each now produces about 50% inhibition; DNP produces 40% inhibition, while the addition of 10 μmoles of ADP again lowers incorporation by 50%. There is no additional inhibition if DNP is added to the ADP containing system, in confirmation of earlier results, and again indicating that incorporation and ATP production appear to be mutually exclusive. Oligomycin stimulates incorporation, especially at low concentrations, an observation not devoid of interest in view of the demonstration by Lee and Ernster (1965) and Ernster and Lee (1964) that oligomycin in this range can also restore various other energy-linked reactions to partially damaged mitochondria. The alternative mode of action for GTP described under (b) appears to be relatively unlikely since GTP-stimulated incorporation appeared to be completely CN^- sensitive and ATP in equimolar amounts did not substitute for GTP. Alternative (d) must be seriously entertained in view of the results obtained with low concentrations of the various nucleoside diphosphates. Of special interest here is the behavior of ADP which greatly enhances incorporation at low (0.1 mM) but inhibits at high (2 mM) concentrations. Perhaps ATP is generated in two different compartments only one of which can be utilized for protein synthesis.

TABLE VIII: Response of Mitochondria in Mannitol Medium without GTP.

Additions	% Standard Activity	
	Experiment A[a]	Experiment B[b]
None	100	100
1.6 μmoles of CN^-	80	74
0.2 μmole of DNP	72	
0.5 μmole of DNP	61	
1.6 mμmoles of rotenone	52	65
2 μg of antimycin-A	48	84
0.05 μg of oligomycin/mg of protein	132	
0.10 μg of oligomycin/mg of protein	108	
0.20 μg of oligomycin/mg of protein	123	
0.50 μg of oligomycin/mg of protein	108	
2 μmoles of ADP	53	
2 μmoles ADP + 0.5 μmole of DNP	45	
No α-KGA	106	
No α-KGA + 2 μmoles of ADP	97	
0.1 μmole of GTP		175
0.1 μmole of ITP		150
0.1 μmole of ATP		107
0.05 μmole of GTP + 0.05 μmole of ATP		119
0.1 μmole of GTP + 1.6 μmoles of CN^-		75
0.1 μmole of GDP		122
0.05 μmole of GDP		136
0.025 μmole of GDP		150
0.1 μmole of IDP		393
0.1 μmole of ADP		708

[a] In experiment A freshly prepared mitochondria were incubated for 1 hr at 37° in air in a medium containing, in micromoles per milliter: 300 mannitol, 0.2 EDTA, 10 Tris pH 7.4, 20 K^+, 10 Mg^{2+}, 10 P_i, 5 α-KGA. In experiment A fresh mitochondria were used; an activity of 100 corresponds to 9.5 μμmoles of radioactive leucine incorporated. [b] In experiment B mitochondria aged for 2 hr were used; an activity of 100 corresponds to 2.8 μμmoles of leucine incorporated.

Response to Substances Affecting Nervous Activity. The compounds studied were the catecholamines, SERO, ACH, GABA, and CPZ. It is quite possible that some neuropharmacological agents owe their activity to an effect on the respiratory cycle, so that the distinction between these drugs and those discussed in the preceding section is not well defined. For example, antidepressive drugs often increase the level of ATP in the brain and decrease that of ADP (Lewis and Van Pelten, 1963). Conversely, tranquilizing drugs may decrease the ATP–ADP ratio (Kaul and Lewis, 1963). Data obtained are presented in Figure 7. Tests were made in two sets of incubation media, with and without ADP.

The effects of neurohormones were to some extent dependent on the test medium. In media containing ADP, EPI at all concentrations stimulated protein synthesis. Maximum stimulation of 35% over standard was obtained at 0.1 mM concentration. However, when ADP was absent in a medium containing P_i, EPI inhibited leucine incorporation by 30%. At lower concentrations EPI had a slight inhibitory effect in both media. With NEPI there was stimulation with ADP–P_i but inhibition with P_i. Dopamine also stimulated in ADP–P_i but inhibited in P_i. Chlorpromazine and SERO, though antagonistic in pharmacological actions, both inhibited leucine incorporation.

The effects of ACH were unusual. There was inhibition in medium P_i, but a remarkable stimulation in ADP–P_i. In fact this experiment, with 1.0 mM ACH, gave the highest rate of leucine incorporation we have so far observed in a mitochondrial system, over

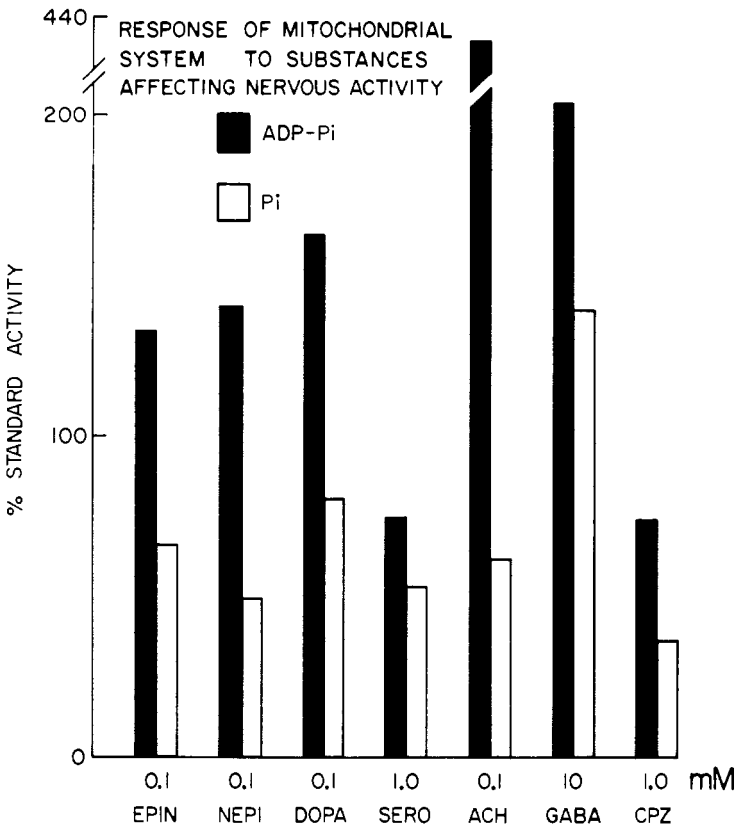

FIGURE 7. Response of [^{14}C]leucine incorporation by mitochondrial system to substances affecting nervous activity.

5000 cpm/mg of protein/hour, corresponding to an incorporation of 66 $\mu\mu$moles of leucine.

One of the most noteworthy effects was that of GABA, which displayed a marked stimulatory action in all systems tested, both mitochondrial and ribosomal.

Discussion

Obviously we do not yet have sufficient information about these two different protein synthetic systems to elucidate their functional significance in the brain. The effects of neurohumoral substances on protein synthetic activity *in vitro* at least do not exclude modes of physiological activity that involve feedback from synaptic transmission to protein synthesis (Briggs and Kitto, 1962).

The influence of GABA on the mitochondrial systems may be connected with its metabolic activity in the brain. Glutamate can undergo decarboxylation to GABA, which in turn is transaminated with α-KGA to yield glutamate and succinic semialdehyde, and the latter compound can enter the citric acid cycle. This process, called the succinic semialdehyde shunt, is known to be operative in brain and about 40% of the oxidation of glutamate is believed to proceed *via* this path (Frankel and Roberts, 1950). Such a mechanism would not be applicable to the ribosomal system, so that some nonspecific effect may be operative there. GABA is a strong dipolar ion and might thus influence electrostatic interactions of proteins and RNA, or between inorganic ions and such biopolymers.

There is a report that a crude preparation of mitochondria from immature rat brain can incorporate a trace of radioactivity from labeled amino acids into a proteolipid fraction similar to that of myelin (Klee and Sokoloff, 1965). Since myelin in developing brain has its source in the oligodendroglia (Nakai, 1963), this case suggests that mitochondria from the glial fraction may be programmed to synthesize myelin. On the other hand, mitochondria from lamb heart are said to synthesize the contractile mitochondrial protein in cell-free systems (Kalf and Grèce, 1964). The situation at present is that *in vitro* mitochondrial preparations may synthesize structural proteins but not enzymes, but *in vivo* mitochondrial enzymes become labeled by added amino acid tracers (Freeman, 1965). Perhaps the enzymes are synthesized outside the mitochondria and migrate in, or perhaps some necessary constituent (m-RNA?) for enzyme synthesis is deleted in the course of isolation of mitochodria. These observations emphasize the importance of extending the analysis of the brain ribosomal and mitochondrial systems to separated glial and neuronal fractions. The latter might be obtained by methods such as that of Rose (1965), but at present the former would require use of tissue culture preparations or possibly material from glial tumors.

Since both mitochondria and synaptic vesicles are

abundant in synaptic regions we should consider the possibility that the structural protein of the vesicle walls may be synthesized by mitochondria. If glial mitochondria can contribute to the synthesis of myelin proteins, it becomes plausible to consider analogous functions for neuronal mitochondria in nerve endings. Also in frog neuromuscular junctions, vesicles have been observed enclosed by membranes, giving a structure difficult to distinguish from that of some terminal mitochondria (Birks *et al.*, 1960). Evidence for such a speculation is that the half-lives of mitochondrial and synaptic-vesicle proteins are both quite long, at least 15 days, as judged from rate of decay of a pulse label of incorporated [^{14}C]amino acids (C. Cotman and K. von Hungen, unpublished data, 1965). A related mechanism would assign to mitochondria the protein synthesis needed to repair and maintain the synaptic membrane, and would suppose that the vesicles are formed from this membrane by a sort of budding or pinocytotic process. The extremely high *in vitro* protein synthetic activity now demonstrated by brain mitochondria inevitably directs our attention to possible consequences of *in vivo* mitochondrial protein synthesis in the nerve end regions.

Although these two systems from rat brain are interesting from the viewpoint of protein synthesis in general, we believe that their greatest value may be found in further applications designed to test hypotheses regarding the functional activity of brain cells and their response to various pharmacological agents.

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