Protein Synthesis by Synaptosomes from Rat Brain

CONTRIBUTION BY THE INTRATERMINAL MITOCHONDRIA

By A. G. HERNÁNDEZ*

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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(1) The characteristics of protein synthesis in microsomal and synaptosomal fractions from rat brain were examined. A high sensitivity to ribonuclease and to cycloheximide, and the need for the presence of pH 5 enzymes distinguished protein synthesis in microsomal fractions from protein synthesis in synaptosomes. (2) Under various conditions of incubation synaptosomal fractions prepared in sucrose showed limited protein synthesis compared with synaptosomal fractions prepared by using Ficoll. Such discrepancies could not be attributed to: (i) animal age, (ii) the metabolic state of the synaptosomal fraction, (iii) the absence of bivalent cations in the incubation medium or (iv) the temperature. (3) Protein synthesis in synaptosomal fractions was inhibited 50–65% by cycloheximide, 38–50% by chloramphenicol, 95% by puromycin, 70% by azide and 40% by deoxyglucose; ribonuclease had only a negligible inhibitory effect. (4) As a first approximation to the localization of the protein-synthetic machinery present in the synaptosomal fraction, the distribution of enzymes and radioactivity in subfractions of prelabelled synaptosomes was determined after osmotic shock with water. Approximately 60% of the total protein synthesis in the synaptosomal fraction occurred in the intraterminal mitochondria. (5) Protein synthesis in the intraterminal mitochondria did not show any fundamental difference from synthesis in somatic mitochondria, with respect to inhibition by cycloheximide and chloramphenicol. (6) It was concluded that if extramitochondrial protein synthesis occurs in synaptosomes, it must be very low.

It has been demonstrated that the atypical protein-synthetic activity of brain mitochondria when incubated *in vitro* in the presence of chloramphenicol (Gordon & Deanin, 1968*a,b*) is most probably due to the presence of cytoplasmic ribosomes entrapped in membranous vesicles of unknown origin (Hernández *et al.*, 1971; Morgan, 1970). To substantiate this theory the characteristics of two other possible contaminating systems, microsomal fraction (Rubin & Stenzel, 1965; Zonzely *et al.*, 1964) and synaptosomes (Austin & Morgan, 1967; Morgan & Austin, 1968; Appel *et al.*, 1969; Austin *et al.*, 1970; Ramírez *et al.*, 1972*a,b*) need to be analysed. Further, the protein-synthetic capacity of the synaptosomes (isolated nerve terminals) is a subject of controversy. It has been proposed that two different protein-synthesizing systems exist in the synaptosomal fraction (Morgan & Austin, 1968; Autilio *et al.*, 1968; Appel *et al.*, 1969; Festoff *et al.*, 1971). Other workers, however, maintain that only mitochondria carry out protein synthesis at the terminal endings (Gordon & Deanin, 1968*a,b*; Bossmann & Hemsworth, 1970; Gordon & Deanin, 1973).

* Present address: Escuela de Biología, Apartado 10098, Universidad Central de Venezuela, Caracas, Venezuela.

The synaptosomal preparations used by different authors have been obtained by using different methods and the capacity of detached nerve terminals to incorporate amino acids into proteins varies greatly (Appel *et al.*, 1969; Autilio *et al.*, 1968; Gambetti *et al.*, 1972; Hernández, 1972). Thus there is some confusion as to whether synaptosomes are able to carry out protein synthesis or not.

The present paper reports the determination of optimal conditions for protein synthesis by nerve terminals isolated on sucrose gradients: fractionation and inhibitor studies have led to the conclusion that the synthesis mainly takes place in the mitochondria and that, under the conditions used, synaptosomal protein synthesis on the whole is very limited.

Materials and Methods

Preparation of homogenate and subcellular fractions

Reagents. L-[³H]Leucine (17 Ci/mmol) and L-[¹⁴C]leucine (344 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Strophantidin G (ouabain) and cycloheximide were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. ADP, ATP, pyruvate kinase, phosphoenolpyruvate and ribonuclease were from C. F.

Boehringer und Soehne G.m.b.H., Mannheim, Germany. Bovine plasma albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Chloramphenicol was from Parke Davis and Co. Ltd., Hounslow, Middx., U.K. All other reagents were A.R. grade.

Tissue homogenates. Male Sprague-Dawley rats (18–20 days old) were killed in the cold by a sharp blow on the neck. The brain was excised within 1 min and chilled in the homogenization medium. The cerebral cortex was separated from the white matter and minced by use of scissors. The mince (1 vol.) was mixed with ice-cold 0.32M-sucrose (9 vol.), except when microsomal fractions were to be prepared. The mince (1 vol.) was then mixed with ice-cold 0.25M-sucrose–25mM-KCl–4mM-MgCl₂–10mM-Tris-HCl, pH 7.4 (4 vol.). Homogenization was carried out in a Potter-Elvehjem-type homogenizer at 0°C with a (Teflon) pestle (diameter 3.2cm, clearance 0.48–0.72mm as measured by the difference of diameters). Six complete strokes of the pestle rotating at 840 rev./min were made; the homogenate was then cooled in ice, rehomogenized as above and kept on ice until required.

Isolation of the microsomal fraction. Cortical homogenates were centrifuged at 12000g for 20 min at 4°C to remove synaptosomes and mitochondria. The microsomal fraction was then sedimented from the supernatant by centrifuging at 100000g for 2 h at 2°C in the Spinco model L preparative ultracentrifuge. The microsomal pellet so obtained was rinsed twice with the isolation medium and drained by inverting the centrifuge tube. This fraction (yield, 3mg of protein/g of fresh tissue) was either used immediately or was stored at –20°C overnight.

Isolation of the pH5 enzyme fraction. This fraction was obtained from the postmicrosomal supernatant (Keller & Zamecnik, 1956) by adjusting the pH to 5 with 1M-acetic acid and centrifuging the cloudy solution at 17000g for 20 min at 0–4°C. The pellet was resuspended in the microsomal incubation medium and used within 24 h.

Isolation of the synaptosome fraction. This utilized essentially the same procedure as Gray & Whittaker (1962). Nuclei, large myelin fragments and cell debris were sedimented from a cortical homogenate at 1000g for 10 min and the pellet was washed twice by resuspension in 0.32M-sucrose and resedimentation at the same speed. The washed pellet (fraction N) was again suspended in 0.32M-sucrose for analysis. The combined supernatants were centrifuged at 17000g for 60 min to give a pellet containing mainly small myelin fragments, synaptosomes and mitochondria. After suspending in 0.32M-sucrose this mitochondrial fraction (P₂) (8 ml) was layered on to 15 ml each of 0.8M- and 1.2M-sucrose which had been left together for at least 1 h before use. The gradient was then centrifuged at 27000 rev./min (60000g)

in the Beckman L2-65B ultracentrifuge SW 27 rotor for 2 h at 2°C. Fractions enriched in myelin (P₂A), synaptosomes (P₂B) and mitochondria (P₂C) separated; these and the relatively clear zones above fraction P₂A (fraction PO) and between fractions P₂B and P₂C (fraction P₂BC) were collected for analysis.

For labelling experiments with the material of fraction P₂B, the synaptosomes were sedimented by diluting fraction P₂B very slowly with an equal volume of sterile cold water, stirring continuously and then centrifuging at 150000g for 15 min at 0–4°C in the L2-65B ultracentrifuge with the no. 50 angle rotor. The synaptosomal pellet was rinsed twice with the isolation medium and drained by inverting the centrifuge tube. The final pellet was suspended in the incubation medium (0.4 ml/g of original tissue) by using a loose glass homogenizer and was used immediately for the incorporation studies.

Subfractionation of labelled synaptosome. Incorporation was stopped by the addition of ice-cold 0.32M-sucrose containing non-radioactive precursor. After centrifugation at 40000g for 30 min at 2°C, the synaptosomal pellet was resuspended in water by use of a tight glass homogenizer. Synaptosomal subfractions were prepared on a sucrose density gradient according to the procedure of Whittaker *et al.* (1964). The various fractions (Fig. 1) were separated by means of a syringe-operated pipette and then either analysed for marker enzymes or treated with trichloroacetic acid to a final concentration of 5% (w/v) for analysis of radioactive protein. Because of the low protein content of some of the fractions bovine plasma albumin (1 mg/sample) was added to each subfraction as a carrier before the addition of trichloroacetic acid, and allowance was made for the presence of this protein when calculating specific radioactivities. The radioactive protein precipitate was collected, washed and counted for radioactivity as described below.

Incorporation studies

Incubation media. The following media were used. (1) Microsomal fraction: samples (1.0 mg of protein) were incubated in a medium similar to that used by Campbell *et al.* (1966) for their ribosomal preparation (microsomal Campbell medium). It contained 0.25M-sucrose, 20mM-Tris-HCl (pH 7.6), 10mM-magnesium acetate, 40mM-NaCl, 100mM-KCl, 6mM-2-mercaptoethanol, 0.05 μM of each of the 20 amino acids except L-leucine, 0.1mM-GTP, 2mM-ATP, 5mM-phosphoenolpyruvate, 10 μg of pyruvate kinase/ml and 7.0–1.0 mg of pH 5 enzyme/mg of microsomal protein.

(2) Synaptosomal fraction: unless otherwise indicated, samples (1.0 mg of protein) were incubated in a medium similar to that used by Autilio *et al.*

Fig. 1. Subfractionation of labelled synaptosomes

The synaptosomal preparation was resuspended in water (2–3 ml/g of fresh tissue) by using a loose homogenizer and then layered on to a density gradient of 5 ml each of 0.4M-, 0.6M-, 0.8M-, 1.0M- and 1.2M-sucrose and centrifuged at 53000g for 2h. In most experiments a cushion of 2 ml of 2M-sucrose was used and the gradient fractionated from bottom to top by means of a syringe device. The diagram shows the relationship between the original gradient and the fractions taken. Banding of particulate material occurred mainly in fractions E, F, G and H with a hazy band in fraction D and a pellet on the bottom of the tube that included fraction I.

(1968) for their synaptosomal fraction (Autilio medium), and contained 0.033M-Tris-HCl, pH 7.6, 0.1M-sucrose, 100mM-NaCl and 10mM-KCl.

Incubation conditions. Subcellular fractions were suspended in the above media and incubated in sterile stoppered vials with air as the gas phase at 30°C for a given time in the presence of the appropriate radioactive amino acid. The vials were shaken at 100 cycles/min in a Dubnoff bath.

Measurements of radioactivity in protein. In experiments on the incorporation of amino acids into proteins, the vials were transferred to crushed ice after the incubation and the contents diluted with the appropriate unlabelled amino acid (2mg/ml) followed by cold trichloroacetic acid to a final concentration of 5% (w/v). The protein precipitate was washed by the method of Simkin & Work (1957) and transferred to an Oxoid membrane (Oxoid Ltd., London E.C.4, U.K.) by filtration, and radioactivity was measured in either a Packard Tri-Carb or a Beckman LS 150 liquid-scintillation counter with toluene scintillation fluid (10ml) containing 4g of

Fig. 2. Effect of pH5 enzyme preparation on brain-cortex microsomal protein synthesis

Microsomal protein (1 mg) in 1 ml of microsomal Campbell medium was incubated with 0.2 μ Ci of [14 C]leucine (344 mCi/mmol) at 30°C for 15 min in the presence of different amounts of pH5 enzyme preparation.

PPO (2,5-diphenyloxazole) and 50mg of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre to an efficiency of 70–85% for 14 C and 10–15% for 3 H. The 14 C channel was used to count 35 S. Allowance was always made for a sample taken at zero time the counts of which were generally very low. The statistical accuracy of counting was between ± 2 and $\pm 5\%$.

Bacterial contamination. All apparatus, media and instruments were sterile. Possible contamination at the end of the incubation was checked by placing a sample (0.1 ml) on blood-agar plates at 37°C. If after 72h more than 200 colonies/ml were found, the results were discarded.

Protein concentration. This was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Protein synthesis by the brain-cortex microsomal fraction

Incorporation studies. Maximal amino acid incorporation was achieved at a ratio of 0.7–1 mg of crude pH5 enzyme protein to 1 mg of microsomal protein (Fig. 2). Similar ratios have been reported by Rubin & Stenzel (1965) and Campbell *et al.* (1966). Zonzely *et al.* (1964), however, found that higher ratios were necessary for optimum amino acid incorporation. These inconsistencies may arise from the different procedures used by these various authors.

The increase in amino acid incorporation by the microsomal fraction in the presence of pH5 enzyme protein is one of the distinctive features of brain-cortex microsomal protein synthesis, since neither the washed P₂ fraction (synaptosomal-mitochondrial

Fig. 3. Detection of 80S ribosomes and polyribosomes active in protein synthesis in brain-cortex microsomal preparations

The microsomal fraction (15 mg), isolated as described in the Materials and Methods section, was suspended in microsomal Campbell medium (5 ml) and incubated at 30°C for 5 min with 3 μ Ci of [14 C]leucine (344 mCi/mmol). The chilled suspension was spun for 2 h; the pellet was resuspended in 0.2 M-sucrose–5 mM-magnesium acetate–10 mM-KCl–10 mM-Tris–HCl, pH 7.6, and lysed by adding Brij-58 (0.1%) and deoxycholate (0.25%), then layered on a linear (15–30%) sucrose gradient in 5 mM-magnesium acetate–10 mM-KCl–10 mM-Tris–HCl, pH 7.6. The gradients were centrifuged at 27 000 rev./min at 2°C for 3.25 h in the Spinco 30 rotor; 1 ml fractions were collected from the bottom of each gradient and scanned for radioactivity (c.p.m.) (●) and E_{260} (○) as described in the Materials and Methods section. The arrow indicates the position of the Krebs cell 80S ribosome used as a marker.

mixture) (Hernández *et al.*, 1971; Hernández, 1972), nor the synaptosomal fraction is stimulated by pH5 enzyme. Subsequent incubations with cortex microsomal fraction were done under optimal conditions (i.e. 1 mg of pH5 enzyme protein/1 mg of microsomal protein). Radioactivity was incorporated into proteins of the microsomal fraction at the initial rate for about 30 min and then ceased.

Incorporation of [14 C]leucine into microsomal proteins was inhibited by cycloheximide (86%), ribonuclease (99%) and puromycin (99%), but it was unaffected by chloramphenicol. The sensitivity to ribonuclease was in contrast with that observed with the synaptosomal-mitochondrial mixture (Hernández *et al.*, 1971) or the synaptosomal fraction, and is further evidence for the existence of membrane-protected cytoplasmic ribosomes in the latter preparations.

The sensitivity of the microsomal fraction to cycloheximide deserves further comment. Cycloheximide has little effect on protein synthesis in brain cytoplasmic ribosomes (Campbell *et al.*, 1966; Gordon & Deanin, 1968a), whereas it is a powerful inhibitor in brain mitochondrial and synaptosomal preparations (Gordon & Deanin, 1968a). It was

assumed therefore that cytoplasmic ribosomes make little contribution to the protein-synthetic activity displayed by their mitochondrial fractions. Although the different concentrations of cycloheximide used in these studies cannot account for the facts, they can be explained by the different proportions of membrane-bound and free polyribosomes present, since Glazer & Sartorelli (1972) have shown that cycloheximide has a higher inhibitory effect on protein synthesis in the former than in the latter polyribosomes. Also, both Campbell *et al.* (1966) and Gordon & Deanin (1968a) carried out their experiments on samples of cytoplasmic ribosomes which had been isolated after treatment with deoxycholate, whereas our experiments were performed on a crude microsomal preparation. Caution is therefore necessary in the interpretation of results obtained with inhibitors of protein synthesis.

Mechanism of protein synthesis by the microsomal fraction. As brain microsomal protein synthesis was sensitive to cycloheximide, it was desirable to demonstrate the presence of 80S ribosomes in the microsomal fraction. After being pulse-labelled for 3 min in the presence of [14 C]leucine, cortex microsomal fractions were fractionated on sucrose gradients (Fig. 3). The gradients yielded a radioactive 80S peak and a polyribosome peak with an S value of 110–115. The remaining radioactivity (approx. 70% of the total applied to the sucrose gradient) was recovered as a pellet at the bottom of the sucrose gradient and was probably polyribosomal in nature. The ratios of E_{260}/E_{280} were about 1.80 for the 80S peak and 1.75 for the 110–115S peak, and were similar to those reported by Murthy & Rappoport (1965) for guinea-pig and rat brain cytoplasmic ribosomes and by Zonzely *et al.* (1966) for rat brain-cortex cytoplasmic ribosomes. The latter authors also found a 56S peak in their cytoplasmic ribosomal profile which is completely absent from my preparations after both radioactivity and absorbance scanning of the sucrose gradients. However, this ribonucleo-protein component was only found when the media in which the ribosomes were suspended and analysed contained very low Mg^{2+} concentrations (1 mM or less) and high K^+ concentrations (100 mM), and it seems probable that it was a breakdown product of the 80S ribosomes.

Protein synthesis by the synaptosomal fraction

It has been demonstrated by enzymic and electron-microscopy studies (Whittaker *et al.*, 1964; Hernández, 1972) that this fraction appears to be sufficiently pure for the subsequent protein-synthesis studies.

Incorporation studies. Since Austin & Morgan (1967), showed that rat brain slices incubated for time periods of up to 1 h incorporated [14 C]leucine

Table 1. Effect of cycloheximide on the distribution of the crude mitochondrial fraction in sucrose density gradients

Two identical batches of crude mitochondrial fraction (P₂) were prepared essentially as described by Gray & Whittaker (1962). The organelle suspensions (7.5 mg of protein) were each incubated in 10 ml of Autilio medium plus 7.0 µCi of [¹⁴C]leucine (344 mCi/mmol) for 15 min at 30°C. In the control experiment (without cycloheximide), 6.25 mg of protein (34395 c.p.m.) was layered on to the gradient. In the cycloheximide experiment 100 µg of cycloheximide/ml (final concentration) and 6.25 mg of protein (4605 c.p.m.) were layered on the gradient. The relative specific radioactivity was obtained by dividing the percentage of radioactivity by the percentage of protein recovered in each fraction.

Fraction	Control expt.					Cycloheximide expt.				
	Protein (%)	Radioactivity (%)	Relative specific radioactivity	Specific radioactivity		Protein (%)	Radioactivity (%)	Relative specific radioactivity	Specific radioactivity	Inhibition (%)
P ₂ A	3.4	6.4	1.89	10600		3.4	3.8	1.1	845	92
P ₂ B	7.5	4.3	0.57	3150		7.4	4.0	0.53	397	88
P ₂ C	64	64	1.00	5430		64.0	64.0	1.00	745	87
Recovery	75	74				75	72

Fig. 4. Time-course of amino acid incorporation into the synaptosomal fraction, incubated in Autilio medium and in medium B

The fraction (1 mg of protein/ml of incubation medium) was incubated with 5 µCi of [³H]leucine (17 Ci/mmol) at 30°C as described in the Materials and Methods section. ○, Synaptosomal fraction obtained on sucrose gradient, incubated in Autilio medium; ●, synaptosomal fraction obtained on sucrose gradient, incubated in medium B (Hernández *et al.*, 1971).

into the synaptosomes in a linear fashion without a time lag, a considerable amount of work has been done on the process of protein synthesis in these structures (Morgan & Austin, 1968; Autilio *et al.*, 1968). The conditions of incubation used by the latter authors were chosen for the incorporation studies because they appeared to be optimal for synaptosomal protein synthesis and also because the composition of the medium is very simple. However, the synaptosomal fractions prepared in sucrose showed a limited activity in protein synthesis when compared with that displayed by the synaptosomal fractions prepared in Ficoll (Autilio *et al.*, 1968), under the same conditions (see below). There are several possible reasons for such differences.

The effect of animal age (Austin & Morgan, 1967) can be discounted since in both their work and the present work animals of the same age have been used. Other factors analysed are listed below.

(1) Metabolic states of the synaptosomal fraction: incorporation of labelled amino acids into synaptosomal proteins could be affected by damage suffered during purification. As an indirect check, fraction P₂ was labelled under conditions where the activity of this preparation in protein synthesis was highly sensitive to cycloheximide and the product was then further fractionated on sucrose gradients. Table 1 shows that only 4.3% of the total labelled protein

Fig. 5. Effect of bivalent cations on amino acid incorporation into the synaptosomal fraction

The fraction (1 mg of protein/ml of incubation medium) was incubated with $3\mu\text{Ci}$ of $[^3\text{H}]$ leucine ($17\text{Ci}/\text{mmol}$) at 30°C as described in the Materials and Methods section. ○, Synaptosomal fraction obtained on sucrose gradients, incubated in Autilio medium; ●, synaptosomal fraction obtained on sucrose gradients, incubated in Autilio medium plus 2mM-MgCl_2 , 2mM-MnCl_2 and 1mM-CaCl_2 (final concentration).

layered on top of the $0.8\text{--}1.2\text{M}$ -sucrose gradient was recovered in the synaptosomal fraction, and the rest was associated with the mitochondrial fraction. This result suggested that, rather than damage to the synaptosomes during isolation on sucrose gradients, it was the relative absence of structures containing entrapped ribosomes that was responsible for the observed low incorporation of radioactivity. These results are in agreement with those of Ramirez *et al.* (1972a). Many difficulties were encountered in attempts to isolate synaptosomes by the method of Autilio *et al.* (1968), and even in the best experiments incorporation by the synaptosomes prepared in Ficoll was only twofold higher than by those prepared on the sucrose gradient (Hernández, 1972). Further, samples prepared by our methods (Fig. 4), under the conditions used by Autilio *et al.* (1968), gave an incorporation on 0.5pmol of $[^3\text{H}]$ leucine/mg of synaptosomal protein after 30min at 30°C , which is similar to that reported by the same group (Gambetti *et al.*, 1972) for an ultrastructural radioautographic study of protein synthesis *in vitro* by synaptosomal preparations from Ficoll gradients. This is in marked

Table 2. Effects of metabolic inhibitors on protein synthesis by the synaptosomal fraction

Synaptosomal fraction (1 mg of protein) in 0.9 ml of Autilio medium + 0.1 ml of aqueous medium was incubated with $5\mu\text{Ci}$ of $[^3\text{H}]$ leucine ($17\text{Ci}/\text{mmol}$) at 30°C for 20 min.

	Radioactivity	
	(c.p.m./mg of protein)	(percentage of control)
Water	1260	100
Cycloheximide ($100\mu\text{g}/\text{ml}$)	440	35
Chloramphenicol ($100\mu\text{g}/\text{ml}$)	856	68
Puromycin (0.5mM)	63	5
Azide (2mM)	378	30
Ribonuclease ($40\mu\text{g}/\text{ml}$)	1135	90
Deoxyglucose (0.1mM)	755	60

contrast with the incorporation reported in their earlier studies of 14pmol of $[^{14}\text{C}]$ leucine incorporated/mg of protein after 20min at 30°C .

Previous results (Hernández *et al.*, 1971; Gambetti *et al.*, 1972), showing that the relative specific radioactivity of the ribosome-containing membrane-bound particles present in their synaptosomal preparation was up to ten times higher than that of the presynaptic ending, support my hypothesis that there is very little, if any, protein synthesis by the synaptosomes and that amino acid incorporation by isolated synaptosomal fractions should be interpreted with care.

(2) The effect of bivalent cations in the incubation medium. In synaptosomal preparations better incorporation is attained in the simple medium of Autilio than in the more complex medium B (Hernández *et al.*, 1971) (Fig. 4). When an Autilio medium, modified by adding (final concentrations) 2mM-MgCl_2 , 2mM-MnCl_2 and 1mM-CaCl_2 (Festoff *et al.*, 1971), was used the incorporation of amino acids into synaptosomal proteins was inhibited (Fig. 5) and aggregates were formed during the incubation process. All subsequent studies were therefore carried out in simple Autilio medium.

(3) The effect of temperature on synaptosomal protein synthesis was investigated. The incorporation of amino acids into synaptosomal proteins during the first 10min of incubation was approximately 25% higher at 37°C than at 30°C , but thereafter the rate of labelling at 37°C rapidly declined, probably because of dilution effects caused by the considerable proteolytic activity that is known to be present in brain preparations. The incubation temperature can therefore be discounted as contributing to the different behavior of the different synaptosomal fractions, and all subsequent studies were carried out at 30°C .

Effect of metabolic inhibitors on synaptosomal protein synthesis. Synaptosomal protein synthesis was

Fig. 6. Effect of ouabain on amino acid incorporation into the synaptosomal fraction

The fraction (1 mg of protein/ml of Autilio medium) was preincubated for 5 min at 30°C in the presence of 1 μ mol of ouabain and then 3 μ Ci of [3 H]leucine (17 Ci/mmol) was added; samples were removed at the times shown. ●, Control; ○, ouabain present at 1 mM final concentration.

inhibited 50–65% by cycloheximide, 38–50% by chloramphenicol, 95% by azide and 40% by deoxyglucose, whereas ribonuclease had a negligible inhibitory effect (Table 2). These results are partially in agreement with those of Autilio *et al.* (1968) and Morgan & Austin (1968); Ramírez *et al.* (1972b) probably used purer preparations than ours and found 89% inhibition by chloramphenicol.

Appel *et al.* (1969) and Austin *et al.* (1970) showed that synthesis by their synaptosomal fractions depended on the operation of the Na⁺–K⁺-activated ATPase* and also that ouabain (0.5 nM) depressed the incorporation of amino acid in a time-dependent fashion. We have confirmed this result with ouabain (Fig. 6) in contrast with work by Ramírez *et al.* (1972b).

Contribution of the intraterminal mitochondria to synaptosomal protein synthesis. This was analysed in two types of experiments.

(1) The localization of the protein-synthetic machinery in these preparations was established approximately from the distributions of enzymes (Hernández, 1972) and radioactivity in prelabelled synaptosomes *in vitro* analysed after osmotic shock

with water in a 0.4, 0.6, 0.8, 1.0 and 1.2M-sucrose gradient (Fig. 1).

Enzyme markers and morphological study (Whittaker *et al.*, 1964; Hernández, 1972) show that monodisperse synaptic vesicles congregate in fraction D, fraction E contains membrane fragments of microsomal dimensions, fractions F and G external synaptosomal membranes, fraction H partially disrupted synaptosomes and fraction I consists largely of intraterminal mitochondria. The clear top band consists of soluble cytoplasmic constituents of the synaptosomes diluted with water and is devoid of particulate material.

Similar experiments were carried out in the presence of labelled amino acids ([3 S]methionine) and inhibitors of protein synthesis (cycloheximide and chloramphenicol) and the distribution of label and inhibitory actions were as shown in Tables 3 and 4 respectively. Two identical fractions of synaptosomes were incubated for 30 min, one with inhibitor and one without (control). No significant differences existed in the percentage of label in the different fractions, whether labelled with leucine or methionine (Table 3). Further, in the three gradients almost 60% of the total amount of radioactivity was associated with the intraterminal mitochondria and the partially disrupted synaptosomal fractions, 14% was associated with the soluble components of the synaptosomal preparations and 10–14% was associated with the synaptosomal membrane fragments that layered between 0.8 and 1.0M-sucrose. Approximately 5% of the radioactivity appeared in the synaptic vesicles, possibly by diffusion of some soluble components belonging to fraction O. From the relative specific radioactivities it can be seen that only those fractions enriched in mitochondria showed a significant increase in label. Qualitatively, chloramphenicol mainly inhibited those fractions enriched in mitochondria, whereas cycloheximide mainly inhibited those enriched in membrane and soluble synaptosomal fractions. Thus it seems that two separate systems are indeed operating in the synaptosomal preparation, one located in the mitochondrion or in the junctional complex (Ramírez *et al.*, 1972b) (sensitive to chloramphenicol) and the other located probably in contaminating structures (sensitive to cycloheximide). This conclusion is supported by our experiments with isolated intraterminal mitochondria (see below) and the work of Hernández *et al.* (1971) in which the existence of 55S ribosomes in synaptosomal mitochondria was demonstrated. The eukaryotic ribosomal system which was thought to be within the synaptosome is thus still open to question.

(2) Isolated intraterminal mitochondria were tested for their capacity to incorporate amino acids into proteins. Protein synthesis did not occur under conditions of oxidative phosphorylation. However,

* Abbreviation: ATPase, adenosine triphosphatase.

Table 3. *Radioactivity distribution in subsynaptosomal fractions of labelled synaptosomal preparations*

In all three experiments, synaptosomal preparations were incubated in Autilio medium for 30 min at 30°C. In Expts. 1 and 2, [³⁵S]methionine (20 Ci/mmol) was used; in Expt. 3, [³H]leucine (17 Ci/mmol) was used. The suspension was freed of medium and lysed with water, then subjected to fractionation in 0.4–1.2M-sucrose (0.2M steps) gradients as described in the text and Fig. 1. In Expt. 1, 19.86 mg of protein (67248 c.p.m. of [³⁵S]methionine) was layered on top of the gradient; in Expt. 2, 16.50 mg of protein (43920 c.p.m. of [³⁵S]methionine) was layered on the gradient; and in Expt. 3, 14.09 mg of protein (18030 c.p.m. of [³H]leucine) was layered on the gradient. All the given amounts were taken as 100% for calculation of the corresponding distribution of protein or radioactivity in each subsynaptosomal fraction. The relative specific radioactivity was obtained by dividing the percentage of radioactivity by the percentage of protein recovered in each fraction.

Fraction	Expt. 1			Expt. 2			Expt. 3		
	Protein (%)	Radioactivity (%)	Relative specific radioactivity	Protein (%)	Radioactivity (%)	Relative specific radioactivity	Protein (%)	Radioactivity (%)	Relative specific radioactivity
O	28.09	14.60	0.52						
D	5.48	3.70	0.67	25.12	14.10	0.56	20	14.02	0.70
E	1.86	1.40	0.76	5.62	4.02	0.71	6.4	5.3	0.81
EF	1.61	1.36	0.84	2.42	1.71	0.70	2.5	1.8	0.72
F	5.03	2.81	0.55	3.25	1.83	0.56	1.4	1.4	0.97
FG	4.73	2.54	0.53	6.60	4.48	0.73	4.3	2.8	0.64
G	10.47	5.53	0.52	6.59	3.18	0.48	3.7	3.0	0.82
GH	6.74	5.01	0.74	13.09	7.24	0.55	9.7	5.8	0.60
H	24.57	26.76	1.08	8.63	4.62	0.53	6.6	5.1	0.78
I	14.50	27.55	1.90	24.96	25.90	1.04	30.8	29.2	0.98
Recovery	103	91		13.76	32.97	2.39	17.0	33.7	1.98
				110	100		102	102	

Table 4. *Effects of chloramphenicol and cycloheximide on distribution of radioactivity in subsynaptosomal fractions*
Expt. 1 was performed with two identical batches of synaptosomal preparations as described in Table 3. In the control one sample containing 19.86mg of protein (67248c.p.m. of [³⁵S]methionine) and chloramphenicol (100µg/ml) was added to the other sample containing 19.56mg of protein (33156c.p.m. of [³⁵S]methionine). In Expt. 2 the same procedure was followed but the second sample contained cycloheximide (100µg/ml). The control sample (16.50mg of protein containing 43920c.p.m. of [³⁵S]methionine) was layered. All the fractions were precipitated with trichloroacetic acid, washed and the specific radioactivities of each fraction determined as described in the Materials and Methods section. Percentage inhibition denotes the % inhibition of each fraction with reference to its respective control fraction. The inhibition by cycloheximide and chloramphenicol of the parent fraction was 50% of the control.

Fraction	Expt. 1			Expt. 2		
	Specific radioactivity		Inhibition (%)	Specific radioactivity		Inhibition (%)
	Control (c.p.m./mg of protein)	Chloramphenicol (c.p.m./mg of protein)		Control (c.p.m./mg of protein)	Cycloheximide (c.p.m./mg of protein)	
O	1759	1513	14	1479	451	70
D	2287	2077	9	2102	750	65
E	2602	2083	20	1507	635	58
EF	2868	2220	23	1520	780	49
F	1890	1512	20	1968	872	56
FG	1819	1334	27	1278	577	55
G	1788	1200	33	1472	803	45
GH	2516	1428	43	1431	1061	36
H	3688	1425	62	2761	2298	17
I	6433	1911	71	6243	5198	17

Fig. 7. *Time-course of incorporation of amino acids into intraterminal brain mitochondrial fraction and the effect of metabolic inhibitors*

The fraction (1mg of protein/mg of ATP-generating medium, Hernández *et al.*, 1971) was incubated with 1µCi of [³⁵S]methionine (19Ci/mmol) at 30°C as described in the Materials and Methods section. When studying the effects of metabolic inhibitors on protein synthesis the following final concentrations of inhibitors were added and the reaction was stopped after 20min of incubation: chloramphenicol (A), 100µg/ml; cycloheximide (C), 100µg/ml; puromycin (B), 2mM.

when an ATP-generating system was used, it was shown that no fundamental differences exist between this fraction and the other mitochondrial populations already studied with respect to inhibition by chloramphenicol (Fig. 7). The kinetics of incorporation of amino acids by the intraterminal mitochondria were very similar to those shown by the purified mitochondria when incubated (Hernández *et al.*, 1971) in the ATP-generating system. Further, protein synthesis was inhibited 90% by chloramphenicol and 99% by puromycin but only 10% by cycloheximide. This fraction also possesses a 50–55S ribosome (Hernández, 1972).

Discussion

It is very unlikely that the microsomal fraction is responsible for the eukaryotic type of ribosomal system which is present either in the ‘crude mitochondrial preparation’ or in the synaptosomal fraction. Thus microsomal protein is absolutely sensitive to ribonuclease and requires the presence of pH5 enzyme protein to carry out the process at optimal rate, whereas protein synthesis in either the synaptosomal fraction or the ‘crude mitochondrial fraction’ is not sensitive to ribonuclease nor does it require pH5 enzyme protein.

The sensitivity of protein synthesis to chloramphenicol by the synaptosomal mitochondria in all the systems tested in the present work supports the idea that intraterminal mitochondria do not differ from mitochondria elsewhere in brain cortex

or liver with respect to the operation of a 50–55S ribosome in mitochondrial protein synthesis.

It is well known that axoplasmic transport is a mechanism that can provide the axons and terminal endings with protein synthesized in the cell body. The present work indicates that it is unlikely that the eukaryotic ribosomal system (probably located within synaptosomes) is responsible, at least in quantitative terms, for the cycloheximide-sensitive system present in my crude mitochondrial fractions, and probably in the synaptosomal fractions of others (Autilio *et al.*, 1968; Appel *et al.*, 1969; Austin & Morgan, 1967; Morgan & Austin, 1968; Festoff *et al.*, 1971).

Anomalous protein synthesis observed in brain mitochondria and synaptosomal preparations is probably caused by a contamination of the preparations by a ribosome-containing particle that contains soluble enzymes and is limited by a plasma membrane. However, the possibility that some proteins in the terminal endings arise from sources other than local mitochondrial protein synthesis or axonal transport cannot be discounted. There is evidence to support the conclusion that if eukaryotic ribosomal protein synthesis occurs at the terminal ending of the neuronal cell its activity is very restricted (Barondes, 1969; Droz & König, 1969).

Similar conclusions were reached by Gambetti *et al.* (1972) in an ultrastructural radioautographic study. They also suggested that another protein-synthesizing system operates in the presynaptic ending *in vitro*, since label was found in radioautographs that contained no mitochondria, and radioactivity not associated with mitochondria was detected when the synaptosomal fraction was incubated in the presence of chloramphenicol. However, the concentration of presynaptic terminal endings that do not contain mitochondria was very low in their preparation, and the characterization of such structures as terminal endings is subject to some bias. Further, no experiment with puromycin (which inhibits protein synthesis by 100%) was reported and the problem of background due to adsorbed radioactive label ($[^3\text{H}]$ leucine) is, in our view, not clearly resolved. Recent demonstrations of rapid axonal transport in the central nervous system (Karlsson & Sjöstrand, 1971) and rapid retrograde transport of proteins in the central nervous system (La Vail & La Vail, 1972) seem to imply that there is no need for local protein synthesis other than that owing to the mitochondria in the terminal ending of the neuron. The results of Ramírez *et al.* (1972b) on the characteristics of the protein-synthetic activity at the junctional complex raise interesting questions about the mechanism and functional significance of such a process. However, the nature of the products synthesized by the brain mitochondria preparations

isolated by Ramírez *et al.* (1972b) differed from those synthesized by rat liver and rat brain mitochondria (Hernández, 1972).

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