AMINO ACID INCORPORATION INTO NERVE ENDING STRUCTURES *IN VITRO*

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1. Introduction

Since the first report by Morgan and Austin [1], a number of papers dealing with the *in vitro* incorporation of labeled precursors into macromolecules of isolated synaptosomes and mitochondria have appeared in the literature [2–15]. Although there is generally good agreement among workers in this field regarding the existence of a peripheral protein synthesizing system, apparently independent of the perikaryal one, the evidence has at times been somewhat ambiguous and contradictory, particularly with respect to the behaviour of the system in the presence of inhibitors of protein synthesis. Accordingly we have attempted to re-examine the problem, using highly purified whole synaptosomes and synaptosomal membranes isolated by a new procedure. The results, together with those of other workers [11], suggest that nerve endings contain at least two different structures capable of autonomous protein synthesis: the mitochondria and the membranes. Both synaptosomal sub-fractions as well as the whole synaptosome appear to incorporate radioactive amino acids in a chloramphenicol-sensitive and cycloheximide-resistant manner.

2. Materials and methods

Synaptosomes and sub-synaptosomal structures were prepared, from rat brain, by a modification of the method of Kornguth et al. [16], which reduces the entire procedure to 24 hr without sacrificing purity. This procedure is described in detail elsewhere [17]. For illustrative purposes, some contamination values are given in table 1.

Incubation media for the different structures, together with other experimental details, are described in the legends to figures and tables.

In all cases the incubation was stopped by addition of an equal volume of 10% trichloroacetic acid containing 10 mM leucine. The precipitate was dissolved in 4 ml of 0.5 N NaOH, kept for 30 min at 45°, and reprecipitated with 1 ml of 4 N trichloroacetic acid. After the pellets were washed with 3 ml of 10% trichloroacetic acid, they were suspended in 3 ml of ethanol/ether (1:1) and incubated at 45° for 30 min. After a final washing with 3 ml of ethanol/ether (1:3), the pellets were dried and dissolved in 1 ml of 1 N NaOH. Aliquots were taken for radioactivity assay in a toluene-based scintillant, containing Triton X-100 (85% efficiency for ^{14}C), and for protein measurement by the method of Lowry et al. [18].

Extreme care was taken to avoid bacterial contamination. All the glassware and instruments were autoclaved, and the solutions were either autoclaved or filtered through 0.45 μm Millipore filters into sterile containers immediately prior to use. Actual contamination was checked by plating 0.1 ml aliquots of the incubated material onto blood-agar plates, just before the end of the incubation.

3. Results and discussion

Our results are displayed in fig. 1 and table 2. Values presented are actual results of one experiment

Table 1
Contamination of synaptosomal membranes by different structures.

Mitochondria	2–5%
Endoplasmic reticulum	3–4%
Myelin	< 2%
Axonal membranes	< 3%
Glial membranes	2–3%

The level of contamination was estimated by assay for appropriate marker enzymes, or by a radioassay described in detail elsewhere [17].

with duplicate samples. More than 6 experiments were carried out under the same conditions with the variation in results, from experiment to experiment, not exceeding 10% for a given set of conditions.

The time course of incorporation of ^{14}C-leucine into isolated synaptosomal membranes, *in vitro*, is illustrated in fig. 1. It can be seen that the incorporation is virtually linear for at least 30 min under the conditions used. It is worthy of note that, although the amount of leucine incorporated is rather low (0.42 picomoles/mg of membranes/hr, when using 1 μCi/ml of ^{14}C-leucine), the incorporation approximately doubles when the amount of precursor available is doubled, indicating that the system is far from saturated. The lower synthetic capacity, in comparison with other preparations described in the literature, may simply be due to the fact that we are dealing with highly purified synaptosomes and synaptosomal membranes, and have eliminated incorporation due to contaminants. Nevertheless the possibility of some damage to the biological material after 24 hr of manipulation, including one step of purification through a gradient of cesium chloride, cannot be excluded.

The effects of some protein synthesis inhibitors on the incorporation of leucine into synaptosomal membranes and synaptosomes are shown in table 2. It can be seen that the membrane incorporation is not affected by cycloheximide or RNase, but is virtually completely inhibited by chloramphenicol. Similarly the incorporation into whole synaptosomes is sensitive to chloramphenicol and completely resistant to cycloheximide. These data may be contrasted with the results of other workers, who have found [1–3, 7, 9, 12] that up to 70% or more of synaptosomal protein synthesis is inhibited by cyclo-

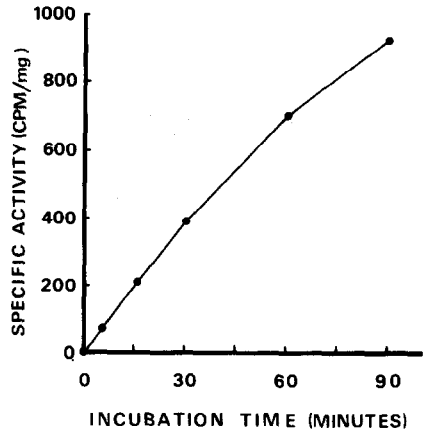

Fig. 1. Time course of ^{14}C-leucine (U) incorporation into isolated synaptosomal membranes, *in vitro*. The incubation was carried out in a 50 ml Erlenmeyer flask containing 10 ml of the following medium: 50 mM Tris-HCl, pH 7.4; 125 mM NaCl; 25 mM KCl; 10 mM $MgCl_2$; 2 mM ATP; 5 mM phosphoenolpyruvate; 50 μg/ml of pyruvate kinase; and 2 μCi/ml of ^{14}C-leucine (U) (The Radiochemical Centre, Amersham, specific activity 344 mCi/mmole). Membrane concentration in this medium was about 2 mg/ml. The incubation was carried out at 37° in a shaking water bath. Aliquots of 0.5 ml were taken, in duplicate, at the times shown in the figure, and processed as described under Materials and methods.

heximide, with only 0–30% being chloramphenicol-sensitive. It is important to note that, in the latter studies, the synaptosomal preparations used were of questionable purity. In general the material has been prepared by the original methods of Gray and Whittaker [19] and De Robertis et al. [20], with some modifications. Although a quantitative estimate of the contamination by other fractions is not usually available, we have found [17] that mitochondria and endoplasmic reticulum are present in the latter preparations to a far greater extent than in our own (see table 1). In addition, Cotman and Taylor [10] have performed autoradiography of synaptosomal preparations, following incubation with tritiated amino acids, and have found that only 48% of the grains are localized over synaptosomes. Similar results were obtained by Gambetti et al. [15]. Accordingly it seems likely that the cycloheximide sensitivity observed in earlier studies on synaptosomal protein synthesis could be due to contamination by 80 S ribosome-containing vesicles, as has been suggested by Morgan [6]. Heavily labeled vesicles of this kind were

Table 2
Incorporation of ^{14}C-leucine (U) into isolated synaptosomal membranes, synaptosomal mitochondria and whole synaptosomes, *in vitro*. Effect of some protein synthesis inhibitors.

Additions	Specific activity (cpm/mg/hr)	% of control
a) Synaptosomal membranes		
None	269	100
Chloramphenicol, 100 μg/ml	28	10.4
Cycloheximide, 200 μg/ml	290	108
Chloramphenicol + cycloheximide (same concentrations)	26	9.7
RNase, 100 μg/ml	250	93
b) Synaptosomal mitochondria		
None	915	
c) Whole synaptosomes		
None	330	100
Chloramphenicol, 100 μg/ml	37	11.2
Cycloheximide, 200 μg/ml	327	99

Incubations were carried out in 10 ml Erlenmeyer flasks containing 2 mg of material in 1 ml of the medium described in the legend to fig. 1. The concentration of radioactive leucine was 1 μCi/ml. The incubation was stopped after 60 min at 37° and specific activity values calculated as described.

identified by Cotman and Taylor in their autoradiographic study [10]. In addition, an elegant study by Hernandez et al. [11] has shown that contamination of mitochondrial preparations by 80 S ribosome-containing vesicles was the origin of the anomalous sensitivity of brain " mitochondria" to cycloheximide [2, 5, 7, 9].

In view of the characteristics of the system, it was of critical importance to determine the contribution to the incorporation into membranes by contaminating mitochondria and bacteria. The synthetic capacity of synaptosomal mitochondria under the present conditions is 3.4 times that of membranes (table 2). Therefore a contamination level of 30% would be required to explain all the incorporation into membranes in terms of contaminating mitochondria. With respect to bacteria, blood-agar plates, containing an aliquot of the incubated material, did not show any visible colonies after 24 hr at 37°. After 72 hr it was possible to count from 50 to 500 small colonies per mg of incubated material (either synaptosomal membranes or whole synaptosomes). These differences between samples with respect to bacterial content were not accompanied by any differences in the specific radioactivity.

Furthermore when *E. coli* cells, growing in the exponential phase, were added to autoclaved membranes deprived of synthetic capacity and the mixture was incubated by the standard procedure, it was necessary to add more than 5,000 bacteria per mg of membranes to get "specific activity" values comparable to those routinely observed. In addition, these fresh *E. coli* gave, after only 24 hr of incubation in blood-agar plates, round colonies up to 3 mm in diameter.

As an additional control, rat optic nerve and tract axonal membranes, free of synaptic endings, were isolated and purified by the same procedure and subsequently incubated under the same conditions as in standard experiments. No incorporation of radioactive precursor was observed although the level of bacterial and mitochondrial contamination was the same as in the synaptosomal membranes.

Weiss has suggested [21] that the disintegration of transported mitochondria, upon arrival at the nerve ending, could provide the material responsible for protein synthesis in isolated synaptosomes. Although our results are consistent with this hypothesis, it is unlikely that mitoribosomal material, by simply sticking to the membranes during the

isolation procedure, could account for the remarkable reproducibility of our specific activity values. Nevertheless such a system may be a means of supplying RNA to the synaptic membranes to aid in the development and maintenance of interneuronal connections.

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