# *In Vitro* Labeling Patterns of Synaptosomal Proteins (37439)

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The association of synaptic activity with specific changes in protein metabolism in the central nervous system has not been achieved with any certainty. Measurement of synaptic proteins in intact animals is limited by such factors as the blood–brain barrier, axonal flow and both anatomic and chemical dilution. To overcome this limitation a number of investigations have focused on synaptosomes, a synapse enriched fraction isolated from brain homogenates (1). *In vitro* synthesis of protein by synaptosomes has been demonstrated in several laboratories (2–5). Sodium and potassium are required for maximum activity and protein synthesis is inhibited by calcium, magnesium and several transmitter substances (6, 7). The site of synthesis and the nature of the proteins synthesized *in vitro* by synaptosomes are unknown, although they appear to be associated primarily with membrane rich fractions (3). We have investigated some of the characteristics of the proteins synthesized *in vitro* using polyacrylamide gel electrophoresis and report our preliminary findings.

*Methods.* Synaptosomes were isolated from 18 to 20 day old rats on a gradient of 5–13–20% Ficoll in 0.32 *M* sucrose as previously described (5). The washed synaptosomal fraction was preincubated for 15 min at 37° with 100 μg/ml RNAse and then incubated in a medium containing 100 m*M* NaCl, 10 m*M* KCl, 100 m*M* sucrose and 33 m*M* Tris buffer (pH 7.6) and 0.5 mg protein. The reaction was initiated by the addition of 10 μCi of $^{14}$C-leucine (316 mCi/mmole) or a mixture of $^{14}$C labeled amino acids (Schwartz-Mann) and carried out for 30 min at 37° in a shaking water bath. The reaction was terminated by the addition of 10 ml of ice-cold 0.32 *M* sucrose. The suspension was centrifuged at 10,000*g* for 30 min and the resulting pellet was suspended in distilled water and kept overnight at 4°. This results in disruption of the synaptosome and gives a water insoluble fraction containing several membranous components (1). Centrifugation was subsequently carried out at 20,000*g* for 60 min, and the resulting pellet solubilized in 1 ml of 1% sodium docedyl sulfate (SDS) to which was added 2-mercaptoethanol and 0.3 *M* iodoacetamide (8). The tube containing this mixture was placed in a boiling water bath for 1 min which resulted in a crystal clear solution. This solution was dialyzed for 48 hr in 0.1 *M* phosphate buffer containing 0.1% SDS and 0.1% mercaptoethanol. Electrophoresis was carried out in 5% polyacrylamide gel containing 0.1% SDS according to the method of Maizel using 150 μg protein/15 cm tube (8, 9). Each run lasted 15 hr at 6.5 mA/tube with migration towards the anode. The gels were crushed in a Savant gel crusher and fractions collected for 20 sec periods resulting in approximately 75 fractions. The crushed gels were incubated with 0.5 ml Soluene for 48 hr at room temperature and counted in a Nuclear Chicago liquid scintillation counter using Bray's solution (10).

For molecular weight determination standard solutions of purified proteins (human gamma globulin, bovine serum albumin, ovalbumin, chymotrypsinogen A and horse myoglobin) solubilized in a similar manner were electrophoresed at the same time as the labeled subfraction (11). When gels were to be scanned 10 cm tubes were used and gels were fixed in a methanol:acetic acid:water (80:7:13) mixture overnight and then stained for 30–45 min in 0.25% Coomassie blue solution. Destaining was in 7% acetic

acid with a short exposure to 50% ethanol solution for final destaining. Migration distances were measured on a lighted viewing box which enabled clear visualization of staining patterns; they were then scanned using linear transport attachment to a Gilford spectrophotometer.

*Results.* The fractionation pattern of labeled protein is shown in Fig. 1A. Eight major peaks were present in all preparations studied with molecular weights as compared to standard proteins ranging between 220,000 and 25,000 daltons. In addition, two smaller peaks were occasionally present, one of high molecular weight and a small peak between those labeled 7 and 8 on Fig. 1A. Although small variations in location of the peaks were noted, the basic pattern was reproducible. Quantitative determinations of the areas below each peak using a planimeter showed a consistent pattern, although the presence of "shoulders" make accurate determination difficult. Table I shows the average area of each of the major radioactive peaks expressed as a percentage of total radioactivity recoverable. Figure 1B shows a scan of a stained gel in which 17 peaks were consistently present, with major peaks having molecular weights between 20,000 and 130,000.

The correlation between spectrophotometric bands and peaks of radioactivity is variable. Peaks 3 and 4 (Fig. 1A) migrate approximately at the same rate as bands M and N (Fig. 1B) while peak 8 which represents 32% of the radioactivity occurs when there are two faintly staining protein bands. Ramirez *et al.* (12) have recently reported that this rapidily migrating peak may represent lipid containing material rather than pure protein.

Several studies of the insoluble protein composition of synaptic membranes have been reported using phenol–urea as the solubilizing agent (13, 14). This mixture does not permit assessment of molecular weight and suffers from the disadvantage of leaving residual protein at the top of the gel. The SDS system used in these experiments has been shown to be reliable in molecular weight determination (15–17), to totally solubilize the synaptosomal fraction, and to result in no staining or radioactive material at the origin. Direct comparisons between these studies are therefore impossible; nevertheless, the previous reports also show approximately 15–16 protein bands which is in agreement with our findings.

TABLE I. Relative Radioactivity of Labeled Peaks.[a]

| Peak no. | % Total $^{14}C$ (±SE)[b] |
|---|---|
| 1 | 3.7 (0.9) |
| 2 | 6.1 (0.2) |
| 3 | 9.2 (0.4) |
| 4 | 8.2 (0.9) |
| 5 | 7.1 (0.3) |
| 6 | 10.2 (1.1) |
| 7 | 18.3 (1.3) |
| 8 | 32.5 (0.9) |

[a] The area under each peak was measured with a Linico planimeter and is expressed as a percentage of total recoverable radioactivity (average of four experiments). Number corresponds to numbered peaks in Fig. 1.

[b] SE = standard error.

The synaptosomal fraction is enriched in presynaptic terminals but may be contaminated with other cellular material. Cottman, Herschman and Taylor (18) have reported that the use of Ficoll minimizes glial contamination and preincubation with RNAase should eliminate cytoplasmic ribosomal contamination. The membrane-bound, ribosome containing particle which has been reported as a contaminant of crude mitochondrial fractions from brain (19), has been observed by us in the fraction sedimenting through 13% Ficoll, but not at the 5–13% Ficoll interface which was used as the source of synaptosomes in these experiments. Deanin and Gordon (20) have recently reported that these particles may contaminate the synaptosomal fraction and suggested that they may serve as a satellite system for protein synthesis in brain.

The nature of the proteins labeled *in vitro* remains unknown. These may be incomplete proteins synthesized only under *in vitro* conditions or they may be related to naturally occurring proteins with high turnover rates. The latter interpretation appears more likely in view of the consistent patterns of molecular

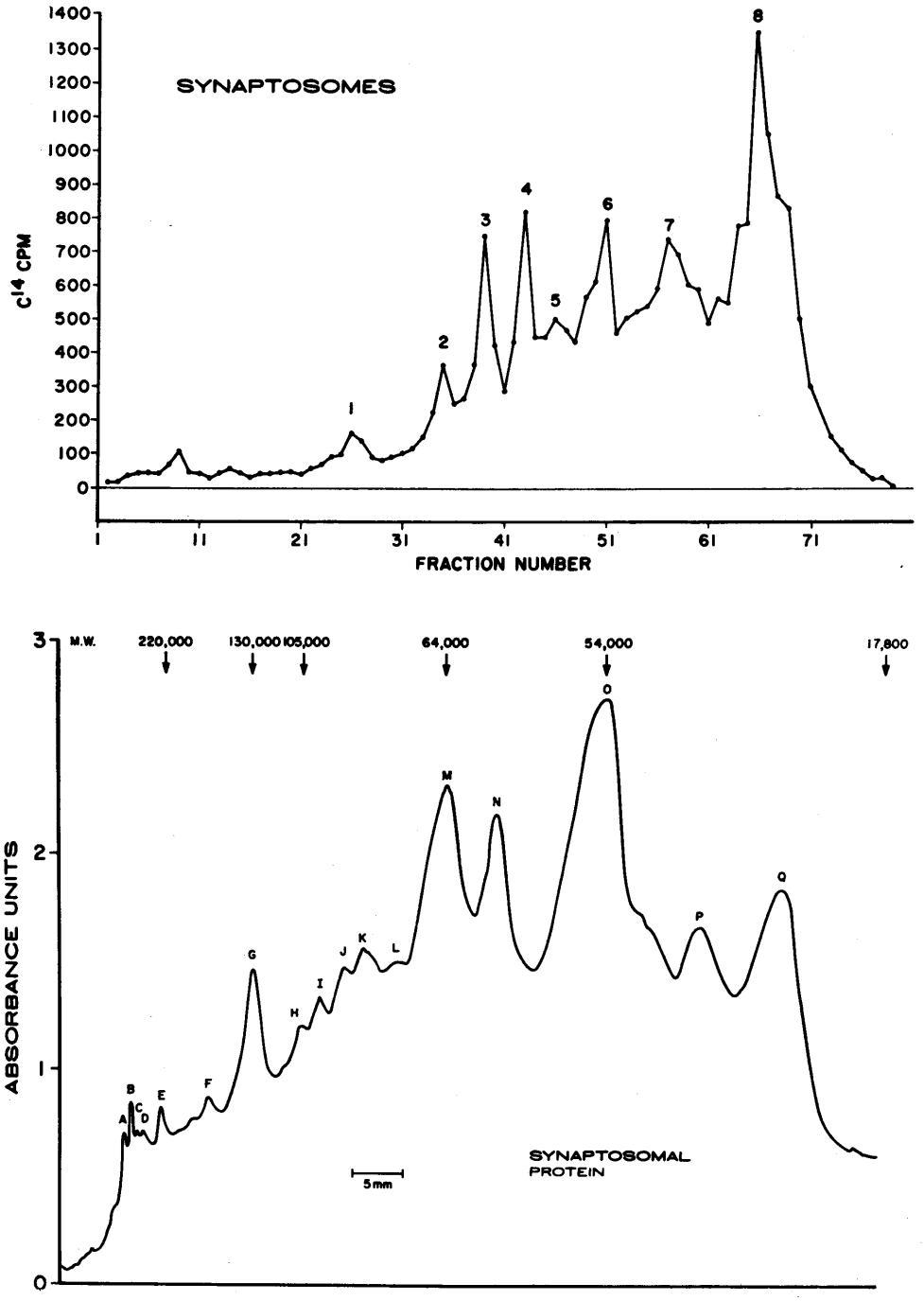


FIG. 1. Insoluble synaptosomal proteins. (A) (top) Radioactivity distribution of a typical 15 cm gel prepared as described in the text. The numbered peaks were always present in the same relative position. (B) (bottom) Linear scan of a 10 cm gel stained with Coomassie blue and scanned at the rate of 0.5 cm/min at 600 nm. Full scale is 3.0 absorbance units. Molecular weights (M.W.) were estimated by comparison with migration of standard proteins of known molecular weight.

weight and relative percentages of total radioactivity observed in all experiments and the approximate correlation between some radioactive peaks and protein bands. The techniques described here, however, do not permit positive identification of the radioactive peaks.

The synaptosomal preparations used in these experiments are not pure synaptic terminals and intrasynaptic mitochondria and perhaps other material may contribute significantly to the observed protein synthesis. However, similar preparations have proven useful in investigations of neurotransmitter uptake and release and the relationship of these activities to protein synthesis is of considerable importance. Other methods which result in more highly purified synaptic membrane preparations cannot be readily correlated with the pharmacologic studies and may result in a highly selected population of synapses which do not reflect *in vivo* activity (21, 22).

*Summary.* Synaptosomes isolated from young rats were incubated with $^{14}$C-amino acids and a water-insoluble fraction obtained which was completely soluble in 1% sodium dodecyl sulfate (SDS). This solubilized, protein containing fraction was subjected to polyacrylamide gel electrophoresis and eight radioactive peaks with molecular weights of 25,000–220,000 were separated. These findings are compared to the protein bands noted in stained gels.

Supported by NIH General Research Support Grant No. RR 05551-10 to the Attending Staff Association of Harbor General Hospital.

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Received Mar. 26, 1973. P.S.E.B.M., 1973, Vol. 143.