THE EFFECT
OF ACETYLCHOLINE ON
RAT BRAIN SYNAPTOSOMAL PROTEIN SYNTHESIS

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ABSTRACT

David Franklin Watson. The Effect of Acetylcholine on Rat Brain Synaptosomal Protein Synthesis (Under the direction of Dr. William James Smith) Department of Biology, July, 1977.

The purpose of this study was to determine the effect of acetylcholine on synaptosomal protein synthesis and the subsequent protein incorporation at the synapse. Past research has shown protein synthesis in the synaptosome and synaptic changes have been suggested as a basis for memory.

Synaptosomes were isolated from the cortex of male Sprague-Dauley rats and incubated with acetylcholine and Eserine for a period of 30 minutes. H^3-leucine was used to determine the amount of new protein synthesized. Specific activity (expressed as CPM/mg protein) was used to compare protein synthesis between the experimental and control. Difference in rates between the experimental and control was expressed as percent change. The data indicated a strong stimulation of protein synthesis in the presence of acetylcholine. There was also indication that only a certain protein fraction in the synapse was effected.

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INTRODUCTION

Historical Review:

Synaptic impulse transmission was once thought to be strictly a matter of electrical conductance. The concept of chemical transmission originated with Elliott(1) in the early nineteen hundreds. This idea received support from the work of Hunt(2) and Hunt and Taveau(3) who discovered the depressant action of acetylcholine while studying the function of adrenal glands. Several years later, Loewi(4) presented evidence for the release of a cardiovascular depressant from a stimulated vagus nerve. This substance, which he called "Vagusstoff," is now known to be acetylcholine. This was the first direct evidence of neurochemical release. We now know that these chemicals (neurotransmitters) are responsible for nerve impulse transmission at the synapse. When the impulse reaches the synapse it causes a quantal release of neurotransmitter. This neurotransmitter affects the postsynaptic membrane in such a way that a new impulse is started.

Protein synthesis and memory. Memory is thought, by some, to involve formation of new nerve circuits. In 1893, Tanzi(5) suggested this was due to changes at the synapse. This change could involve synthesis of new protein or modification of existing protein molecules. Researchers, such as Hyden, Telwar, and Rose, have examined the possibility of these neuronal changes. Hyden(6) showed a larger uptake of H^3-leucine into the Hippocampus of trained rats than into the Hippocampus of the untrained control group. Telwar, et al.(7) and Rose(8) showed increased incorporation of labeled amino acids into the visual

areas of the brain following illumination. Furthermore, several brain-specific proteins have been isolated(9). RNA changes have also been indicated in memory formation(10).

Recent work has involved locating the site of neuronal protein synthesis and incorporation. It was once thought, since the nucleus is in the cell body, that all protein was synthesized in the nerve cell body and transported to nerve endings via axoplasmic transport. However, Autilio, et al.(11) and Austin, et al.(12) have shown protein synthesis within isolated nerve endings called synaptosomes.

Synaptosomal ultrastructure. When brain tissue is homogenized under controlled conditions, the presynaptic nerve terminal pinches off and its enclosing membrane reseals. Whittaker called this a synaptosome(13). Jones later expanded this definition to include the junctional material and attached postsynaptic membrane fragments(14).

The synaptosome has most of the structures found in the in situ synapse. Jones went as far as to call them "structurally accurate representations of the in situ synapse(14).

The presynaptic component is the most complete part of the synaptosome. The presynaptic vesicular grid, found at the synapse, is composed of a series of triangularly arranged dense projections connected by thin filaments (fig. 1). A monolayer of synaptic vesicles is arranged hexagonally around the dense projections(14), (see also fig. 1 & 2). A hexagonal array of synaptopores has also been described by Whittaker(15), (see also fig. 1). A diffuse and irregularly arranged presynaptic network lies in a large area of this presynaptic component(14), (see also fig. 1). There is also an additional membrane coat on the presynaptic

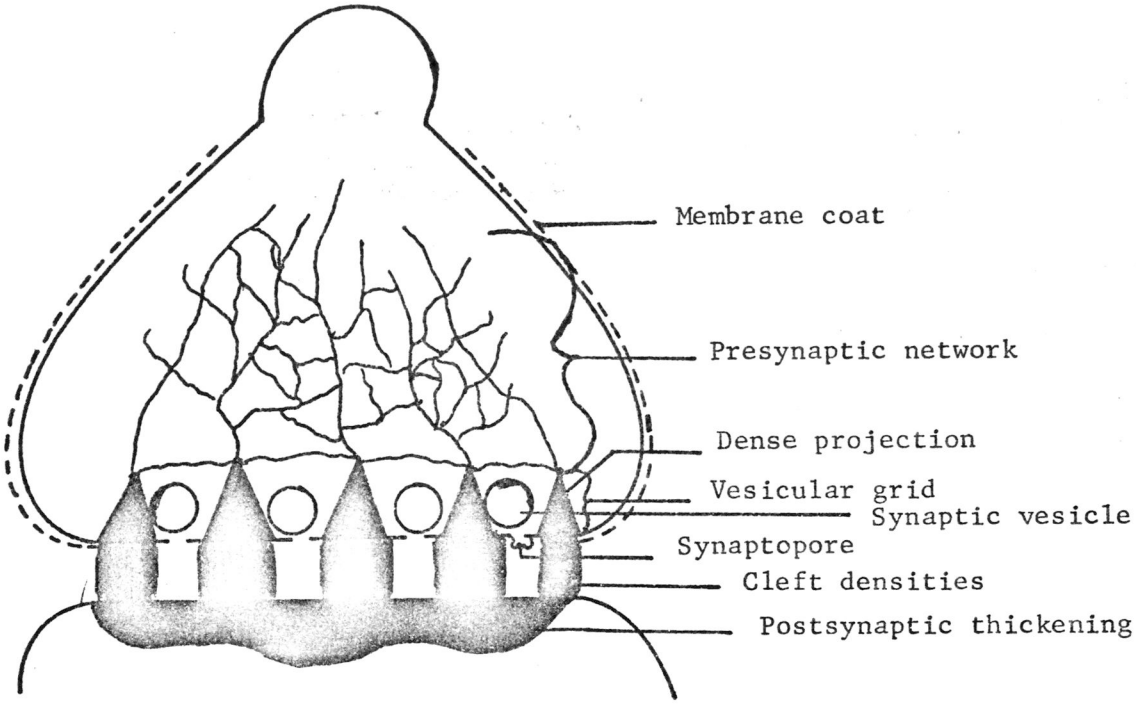


Figure 1. Diagram of Synaptosome

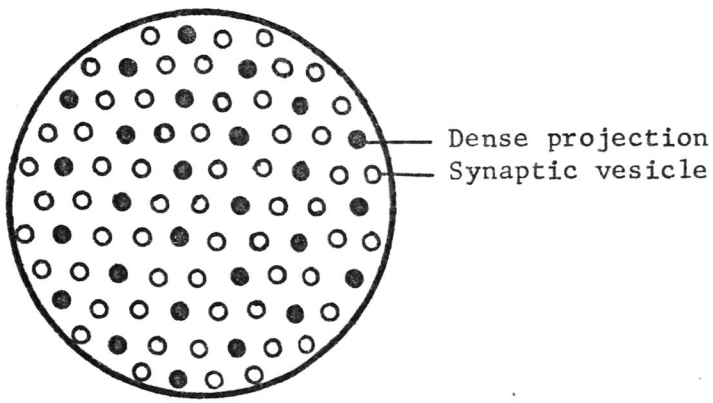


Figure 2. Arrangement of Synaptic Vesicles Around Dense Projections

component(14), (see also fig. 1). The material separating the pre- and postsynaptic membranes takes the form of cleft densities(14).

The postsynaptic membrane has a postsynaptic thickening that is continuous along its length and extends the width of the synapse(14), (see also fig. 1).

Physiological mechanisms. The presynaptic component has all of the substances essential for respiration, including mitochondria and glycolytic enzymes(14). Synaptosomes are capable of taking up potassium and extruding sodium against a concentration gradient, generating ATP and phosphocreation, and linear respiration in the presence of glucose(14). Bradford found respiration to continue linearly for three to four hours and he found little structural change even after 24 hours incubation(14). Essentially synaptosomes are, in the words of Whittaker, "miniature nonnucleated cells"(14).

Protein synthesis in synaptosomes. In addition to respiratory mechanisms, substances essential for protein synthesis have also been reported in synaptosomes. Shephard(16) reported ribosomes, but they are rare, and Austin, et al.(12) reported amino acid activating systems.

Autilio, et al.(11) reported linear uptake of labeled leucine for a period of about thirty minutes. Their work with various ions and energy sources showed synaptosomal protein synthesis to be independent of exogenous energy sources and very dependent on sodium and potassium ion concentrations. Inhibitor studies showed synaptosomal protein synthesis to be mainly eukaryotic. The work of Austin, et al.(12) supported these studies.

Pittman, while working with whole brain slices, recently reported

stimulation of protein synthesis by acetylcholine in certain areas of the brain(17).

Purpose:

Most of the neuronal protein synthesis studies so far have been concerned with general protein incorporation in the entire intact synaptic plasma membrane. Many of the neurotransmitter studies, such as that of Pittman(17), have used whole brain slices as experimental models. The present study differs from others since it concerns the effect of acetylcholine on protein synthesis in isolated synaptosomes with emphasis on synthesis of synaptic complex proteins. The synaptic complex is composed of the material intimately associated with the synapse including cleft densities and attached pre- and postsynaptic membrane fragments. Isolated synaptosomes were used as experimental models because a direct effect of the acetylcholine could be studied free of indirect effect via the nucleus of the neurons. The synaptic complex proteins were chosen for protein incorporation study because many current memory theories involve synaptic facilitation via synaptic membrane protein.

MATERIALS AND METHODS

Reagents:

All common chemicals were obtained from Fisher Scientific Company. Ficoll and Triton X-100 were obtained from Sigma Chemical Company. L-leucine- 4,5-H^3 was from New England Nuclear. PPO* and dimethyl-POPOP** were obtained from Packard.

All 0.32M sucrose solutions were adjusted to pH7.0 with a small amount of 1.0N sodium hydroxide. The first incubation medium was prepared according to Austin, et al.(12) and consisted of 300mM sucrose, 0.2mM EDTA, 10mM potassium phosphate(monobasic), 5.0mM magnesium chloride, and 10mM Tris (ph7.4). The second incubation medium was prepared according to Autilio, et al.(11) and consisted of 33mM Tris-Cl buffer (ph7.6), 100 mM sucrose, 100mM sodium chloride, and 10mM potassium chloride.

The scintillation fluid consisted of 0.82% PPO and 0.025% dimethyl-POPOP in toluene mixed 2:1 with Triton X-100.

Isolation of Synaptosomes:

Male Sprague-Dauley rats weighing approximately 500-600 grams were lightly etherized, decapitated, and the cortex was removed. Synaptosomes were then isolated according to Cotman(18), (see also fig. 3).

The cortex (20% w/v) was immediately placed in 0.32M sucrose at 4°C and homogenized at about 400RPM for eight passes in a size C (50ml)

*PPO is 2,5-diphenyloxazole
**dimethyl-POPOP is 1,4-bis- [2,(4-methyl-5-phenyloxazolyl)] -benzene

Thomas glass-teflon homogenizer. All subsequent steps, up to lysing of synaptosomes, were performed at 4°C. The homogenate was diluted to 10% w/v in 0.32M sucrose, divided into two fractions in 40ml centrifuge tubes, and centrifuged at 1,000xg for five minutes in a Sorvall RC-3 centrifuge using a swinging bucket rotor. The sediment containing the nuclear fraction was discarded and the supernatant was centrifuged at 11,000xg for 20 min in a Sorvall RC2-B centrifuge using an SS-34 slant-head rotor and 40ml centrifuge tubes. The supernatant was discarded and each of the mitochondrial pellets obtained were then resuspended using a size A (10ml) Thomas glass-teflon homogenizer and washed twice in 12ml of 0.32M sucrose. Care was taken in decanting the supernatants from the two washes since the resulting pellets were loosely packed.

After the second wash, each pellet was resuspended in 5ml of 0.32M sucrose and equally divided for layering on three Ficoll-sucrose gradients. The volume needed to fill the tubes (approximately 9ml) was used to wash remaining material from the homogenizer. The six Ficoll-sucrose gradients consisted of 5ml each of 8.5%, 13%, and 17% w/v Ficoll in 0.32M sucrose. These were placed in an SW27 swinging bucket rotor and centrifuged at 68,500xg for 45 min in a Beckman L2-65B ultra-centrifuge.

The 0.32M sucrose-8.5% Ficoll fraction and the pellet were discarded. The remaining fractions, consisting of 70% synaptosomes, from each tube were placed in separate 40ml Sorvall tubes, filled with 0.32M sucrose, and centrifuged at 18,000xg for 20 min using the SS-34 slant-head rotor.

Incubation:

The incubation medium of Austin, et al.(12) was used in the first

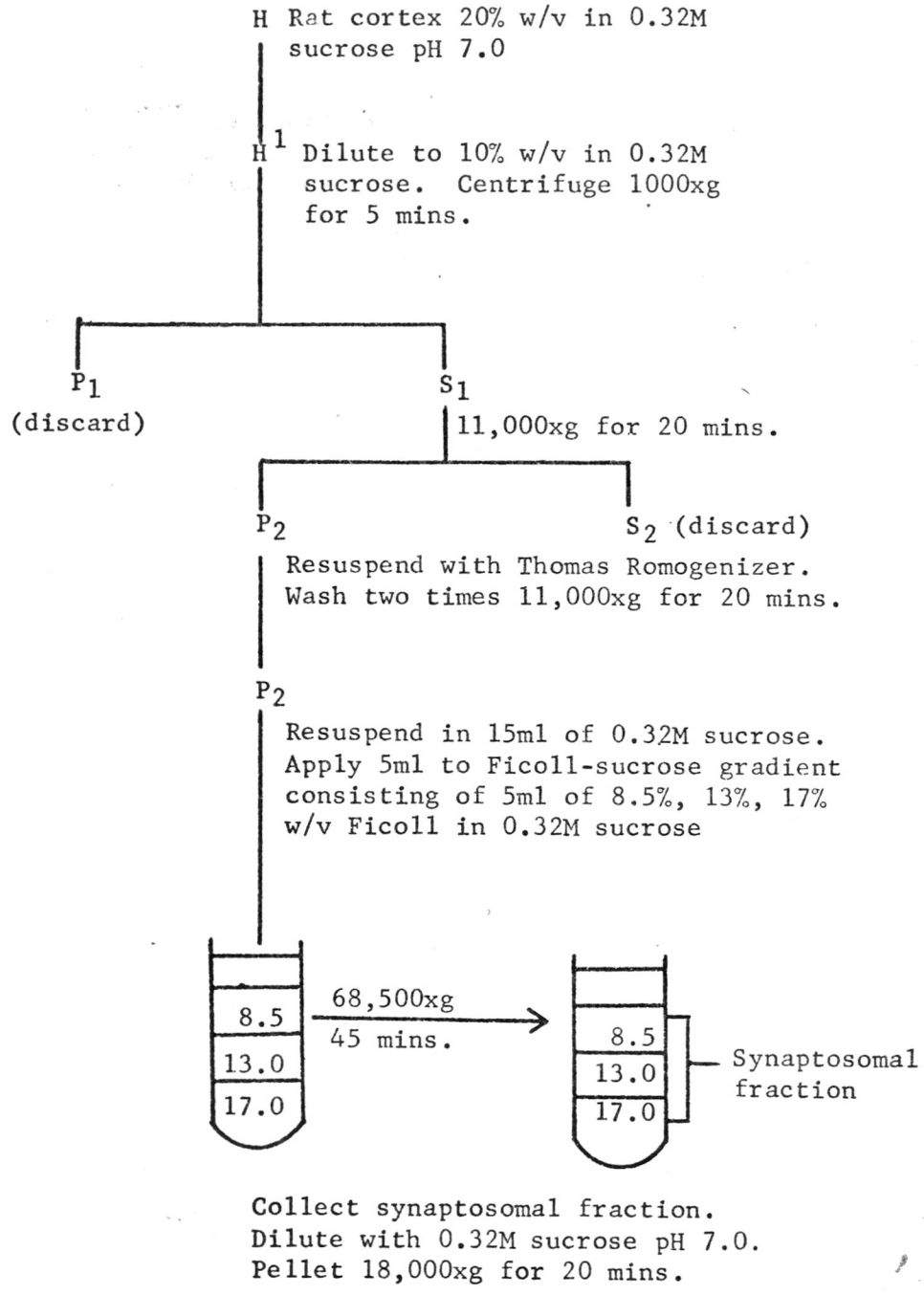


Figure 3. Isolation of synaptosomal fraction from rat cortex. Taken from Cotman (21).

13 experiments and the medium of Autilio, et al.(11) was used in the last four.

After carefully decanting the supernatant, the pellets were carefully resuspended with a glass stirring rod in 4.5ml of incubation medium and stirred with an automatic stirrer to remove any material stuck to the sides of the tubes. The suspensions were mixed and added to Warburg manometer flasks. Both the control and experimental flasks contained 0.1ml of 10mM potassium hydroxide in the center well and 0.1ml H^3-leucine in the main body of the flask. In addition, the control had 2.9ml synaptosomal suspension and the experimental had 2.3ml synaptosomal suspension, 0.3ml of 10mM acetylcholine, and 0.3ml of 0.1mM Eserine to make the final volume of each flask 3.1ml. After a five minute equilibration period in a 37°C water bath, the acetylcholine and Eserine, contained in the side arm, were mixed with the synaptosomes and the contents incubated 30 min.

After incubation, the contents were again pelleted at 11,000xg for 20 min using a 10ml Sorvall tube in the SS-34 slant-head rotor. To remove exogenous H^3-leucine, each pellet was washed three times in 10ml of 0.32M sucrose. Between washes, the homogenizer used for resuspension, was rinsed ten times with water and once with sucrose. The sucrose rinse helped prevent inadvertant lysing of synaptosomes.

Collection of Synaptic Plasma Membranes:

Cotman's(18) procedure was used to collect and purify membranes (see also fig. 4). After the three washes, each pellet was resuspended in 1.5ml of 0.32M sucrose, placed in a 30ml ultracentrifuge tube, and

filled with a cold 50 μM calcium chloride lysing solution. After 15 min at room temperature, each pellet was homogenized at 400 RPM for three passes. This was then pelleted at 100,000xg for one hour, using the 60 Ti slant-head rotor. Each pellet was resuspended in 0.32M sucrose, containing 50 μM calcium chloride, and divided evenly between three gradients. The volume needed to complete the gradients was used to wash remaining membranes from the homogenizer used in resuspension. The gradients consisted of 5ml each of 0.7M, 1.0M, and 1.2M sucrose. Each solution contained 50 μM calcium chloride. These were centrifuged at 68,500xg for 1.5 hours using the SW27 swinging bucket rotor.

The 0.7M-1.0M fraction collected from the three gradients was placed in a 30ml ultracentrifuge tube, filled with lysing solution, and pelleted at 100,000xg for one hour using the 60 Ti rotor.

Isolation of Synaptic Complexes:

Each of the previous pellets were treated with Triton X-100 according to Cotman and Taylor(19) (also see fig. 5). It was previously determined in our lab that 2.9 grams wet-weight cortex yields two pellets approximately 0.36mg each. This value was used to find a 1:1 Triton to protein ratio. After resuspending the pellet in 0.5ml of 2mM Tris (pH7.5), the Triton was slowly added while stirring the suspension. The treated suspension was allowed to sit ten minutes at room temperature and was then layered on 1.0M sucrose, containing 50 μM sucrose, in a 10ml ultracentrifuge tube. The tubes were then centrifuged at 68,000xg for 1.25 hours in a type 40 slant-head rotor.

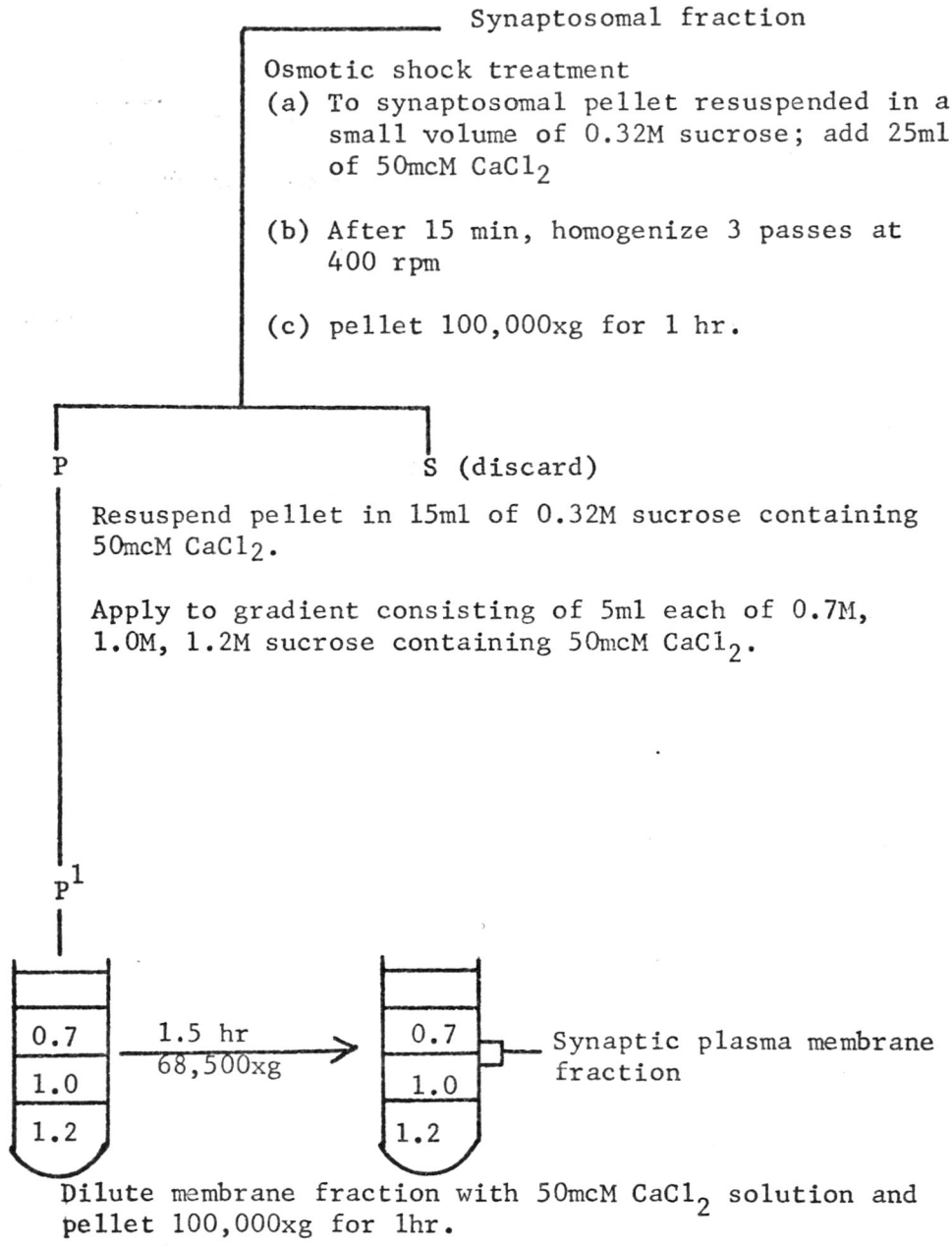


Figure 4. Isolation of synaptic plasma membrane fraction from synaptosomal fraction. Adapted from Cotman (21).

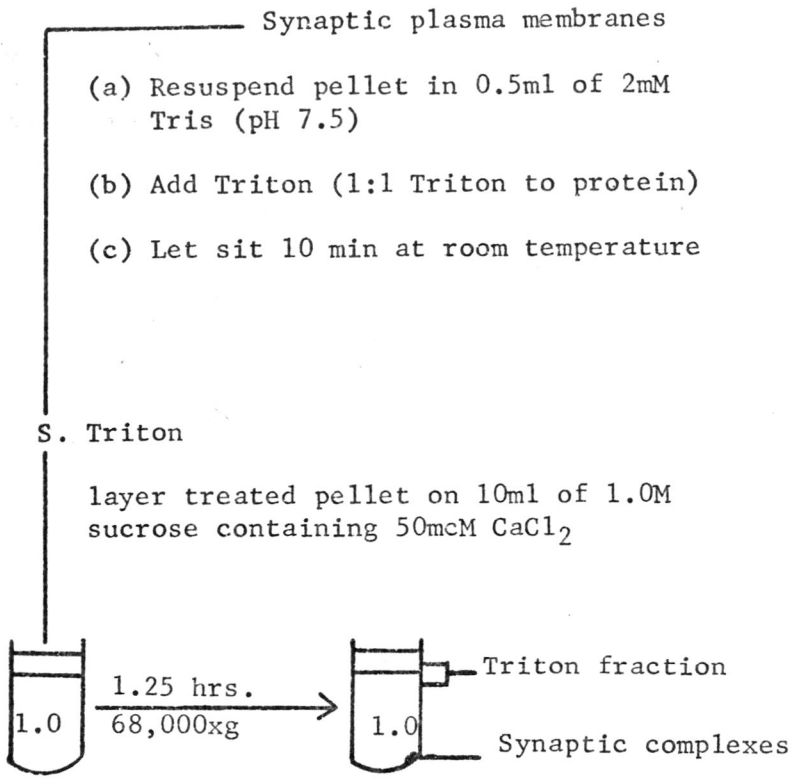


Figure 5. Isolation of synaptic complexes from synaptic plasma membrane fraction. Taken from Cotman and Taylor (22).

Protein Determination:

The resulting pellet was dissolved in 0.2ml of 10% sodium dodecylsulfate (SDS). Total protein in 0.1ml was determined by Lowry's spectrophotometric method.

Radioactivity Measurement:

The remaining 0.1ml of dissolved protein was added to 10ml of Triton-toluene-PPO-dimethyl POPOP scintillation fluid. This was counted 100 min in a Packard Liquid Scintillation Spectrophotometer.

Treatment of Data:

Counts per minute per milligram protein were obtained for the experimental and control samples. The data was compared using the following formula: $\sqrt{[(\text{Experimental}-\text{Control})/\text{Control}]} \times 100 = \%$ Stimulation. A positive value indicated stimulation. A negative value indicated inhibition.

RESULTS

In the initial thirteen experiments, Austin's(12) incubation medium was used. Acetylcholine was found to increase synaptic protein synthesis by an average of 51% as shown in Table 1. The percent change ranged from-37% to +245%.

Later a second medium, reported by Autilio, et al.(11), was tested in the absence of acetylcholine and was found to increase protein synthesis 39% over Austin's medium, as shown in Table 2, so Autilio's medium was chosen for the remaining experiments. In Autilio's medium the average stimulatory effect of acetylcholine in four experiments was increased to +76% as shown in Table 3. The stimulation values ranged from +17% to +157% in these experiments. An overall average stimulation of +57% was determined using the two individual incubation media in the presence of acetylcholine.

Figure 6 is another representation of the results from the two incubation media. Percent stimulation is expressed as a function of final pellet yield in milligrams protein. All of the inhibitory effects, except one, and most of the mildly stimulatory effects occur at a protein concentration below 0.0125mg. Also, the results obtained in Autilio's incubation medium are more positive than those obtained in Austin's medium at comparable protein concentrations.

In early experiments, Triton-treated material layering above 1.0M sucrose was collected as the Triton fraction and analyzed. Results varied from -59% to +94% and averaged +23% as shown in Tables 4 and 5. Since no pattern could be distinguished, collection of the Triton fraction was discontinued.

Table 1. The Effect of Acetylcholine on Protein Synthesis Using Austin's Incubation Medium

Experiment	Radioactivity, CPM		Protein, mg		Specific Activity cpm/mg protein		% Change
	Experimental	Control	Experimental	Control	Experimental	Control	
1	1105	751	0.0355	0.0345	31,130	21,770	+ 43
2	357	126	0.021	0.023	17,000	5,480	+210
3	394	300	0.034	0.036	11,590	8,330	+ 39
4	167	93	0.0165	0.013	10,120	7,150	+ 42
5	90	57	0.024	0.006	3,750	4,390	- 15
6	76	22	0.006	0.015	12,670	3,670	+245
7	662	235	0.0215	0.009	30,790	15,670	+ 97
8	214	243	0.0125	0.0125	17,120	27,000	- 37
9	640	1185	0.0085	0.0075	75,290	94,800	- 21
10	626	497	0.0075	0.005	83,470	66,270	+ 26
11	190	187	0.006	0.057	31,670	37,400	- 15
12	263	186	0.059	0.011	4,460	3,260	+ 37
13	441	454	0.0095		46,420	41,270	+ 13

Average = + 51

Tabel 2. The Effect of Autilio's Incubation Medium Vs Austin's Incubation Medium

Radioactivity, CPM		Protein, mg		Specific Activity cpm/mg protein		
Autilio	Austin	Autilio	Austin	Autilio	Austin	% Change
571	488	0.0105	0.0125	54,380	39,040	+ 39

Table 3. The Effect of Acetylcholine on Protein Synthesis Using Autilio's Incubation Medium

Experiment	Radioactivity, CPM		Protein, mg		Specific Activity cpm/mg protein		
	Experimental	Control	Experimental	Control	Experimental	Control	% Change
14	60	49	0.0065	0.008	9,230	6,130	+ 51
15	19	10	0.0065	0.004	2,920	2,500	+ 17
16	133	63	0.0905	0.0755	1,470	830	+ 77
17	51	23	0.0305	0.0355	1,670	650	+157

Average = + 76

Table 4. The Effect of Acetylcholine On Protein Synthesis In The Triton Fraction Using Austin's Medium

Experiment	Radioactivity, CPM		Protein, mg		Specific Activity cpm/mg protein		% Change
	Experimental	Control	Experimental	Control	Experimental	Control	
1	405	572	0.049	0.056	8,270	10,210	- 19
2	369	294	0.0325	0.0285	11,350	10,320	+ 10
4	118	248	0.029	0.025	4,070	9,920	- 59
5	171	73	0.046	0.037	3,720	1,970	+ 89
6	45	32	0.009	0.008	5,000	4,000	+ 25
7	2,441	650	0.0715	0.037	34,140	17,570	+ 94

Table 5. Comparison of % Stimulation Between Synaptic Complex and Triton Fractions

Experiment	Complexes	Triton
1	+ 43	- 19
2	+210	+ 10
4	+ 42	- 59
5	- 15	+ 89
6	+245	+ 25
7	+ 97	+ 94
Average	+104	+ 23

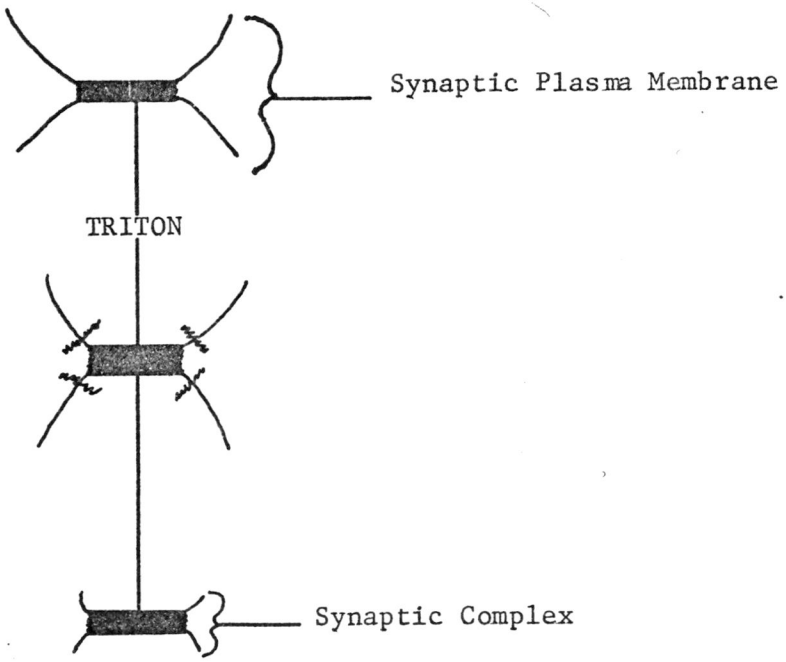
DISCUSSION

Yield presented a major problem in this work. As can be seen in Tables 1 & 3 and in Figure 6, there was a wide variation in yield during the course of the experiments. Two probable sources for this variation would be the purification procedures and the initial homogenization.

Relatively pure fractions were considered very important so purity was chosen over yield in experimental design. This presented a problem though because each purification step meant a potential loss in material. The mitochondrial pellet washings and the pelleting of the material from the Ficoll gradients provided relatively loosely packed material. This further increased the possibility of loss, and variation of yield, through decanting of the supernatant. Variation here can be minimized with practice.

The second probable source of variation involved the initial homogenization and the way it affected the final Triton treatment. Homogenization speed needed to remain relatively constant from one experiment to the next since decreased yield could have occurred with underhomogenization and overhomogenization. Our system didn't allow precise speed control from one experiment to the next. This, in addition to other factors mentioned, may have contributed to variation in size of the final Synaptic Plasma Membrane (SPM) fraction. This fraction was usually very small (less than 0.5mg) so any variation could have made a difference in regard to treatment with Triton. The SPM fraction was treated with a 1:1 (w/w) ratio of Triton to protein. The size of each SPM fraction was estimated from a previously determined value (see

Material and Methods: Isolation of Synaptic Complexes). The size of the SPM fraction was critical since addition of too much Triton severely damaged synaptic complexes and addition of too little prevented total separation of the complexes from the SPM. Triton released the complexes according the following scheme.



Homogenization should be performed with a machine that will operate at constant speeds from one experiment to the next. This will allow a uniform SPM fraction which can be measured and used to determine the amount of Triton added. An alternate procedure is to run an additional control to be used strictly for measuring pellet size.

The variation in percent stimulation might be puzzling unless examined in the light of Figure 6. This figure shows a large variation in percent stimulation between those with a low protein recovery and those with a higher recovery. If the one high value of percent stimulation (+245) at protein concentrations below 0.0125mg is eliminated, then the average of all those below 0.0125mg approaches zero. Even including the high value (+245), the average of the low recovery group is +30% compared to +76% for the high recovery group. The low recovery group values could be due to several factors including insufficient protein for accurate measurement and unequal release of synaptic complexes. A curve is drawn on Figure 6 that shows a trend indicating that in many experiments the amount of Triton is insufficient or in excess.

The strong stimulation obtained not only supports the work of Autilio, et al.(11) and Austin, et al.(12) who have shown protein synthesis to be partially independent of the nerve cell body and axon, but it also raises several questions. How is the protein synthesized? Where is it incorporated? Where does the acetylcholine have its effect?

The scarcity of ribosomes poses a problem in regard to how and where the proteins are synthesized. The proteins may be due to contamination by mitochondria. However, the inhibition studies of Austin, et al.(12) and Autilio, et al.(11) help rule out this possibility. Austin, et al.(12) have also reported the presence of amino acid activating systems and RNA which they suggest may be ribosomal in nature.

The protein may be synthesized anywhere including the postsynaptic membrane. However, it is likely synthesis occurs in the presynaptic

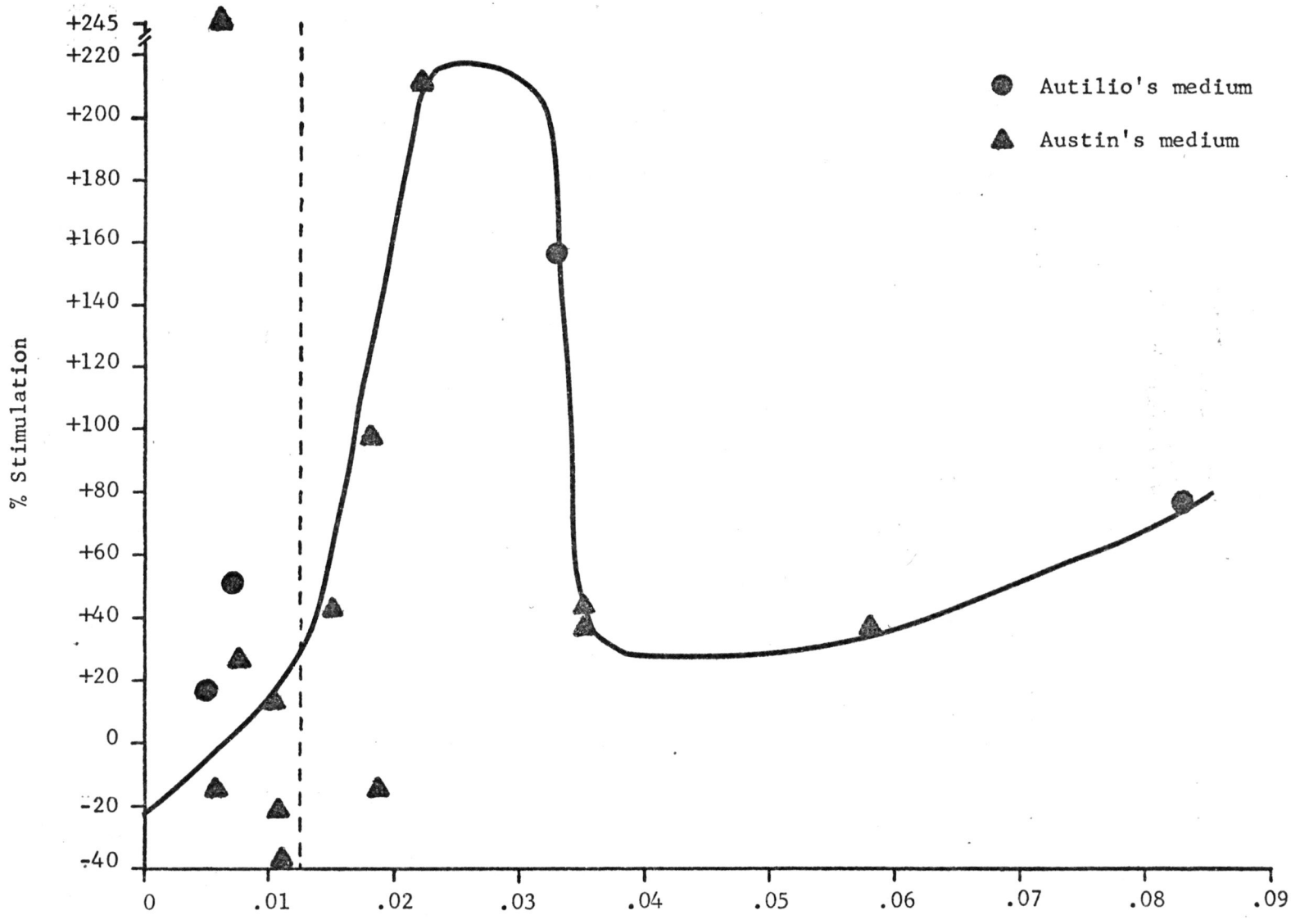


Figure 6. Relationship between % stimulation and final synaptic complex yield.

component. Protein synthesis has been shown to be independent of exogenous energy supplies and the respiratory mechanisms are associated with the presynaptic component. Synthesis in the presynaptic component also helps explain increased synthesis with Autilio's(11) incubation medium. Autilio's medium contains sodium and potassium which are essential for nerve function.

If protein is synthesized in the presynaptic component, then the effects of acetylcholine are probably initiated here. This would offer partial support to Koelle's theory of synaptic transmission. He says initial acetylcholine release reacts with the presynaptic membrane to cause further neurotransmitter release. This secondary release affects the postsynaptic membrane.

The protein synthesized may be incorporated anywhere within the SPM. The results in Table 5 indicate that most of the newly synthesized protein is incorporated in one particular region instead of being dispersed throughout the SPM. This particular region seems to be the synaptic complex.

Conclussion:

This work has shown an increase in synaptic membrane protein synthesis in the presence of acetylcholine. The major site of incorporation was the synapse. This increased synthesis may be a form of synaptic facilitation due to acetylcholine. These results provide a specific site to search for proteins possibly involved in the memory mechanism. However, much work will have to be done before any link can be made between this work and memory.

BIBLIOGRAPHY

1. Elliot, T. R., J. Physiol., 31, 20-21 (1904).

2. Hunt, R., Amer. J. Physiol., 5, 6-7 (1901).

3. Hunt, R. and Taveau, R. deM., Brit. Med. J., 2, 1788-1791 (1906).

4. Loewi, O. In: Hugh McLennan, Synaptic Transmission, W. B. Saunders Company, Philadelphia, 25 (1970.

5. Tanzi, E., Riv. Sper. Freniat., 19, 149 (1893)

6. Hyden, H. "Some Brain Protein Changes Reflecting Neuronal Plasticity at Learning," In: Karczmar, A. G. and Eccles, J. C. (eds.), Brain and Human Behavior, Springer-Verlag, New York, 94-111 (1972).

7. Telwar, G. P., Chopra, S. P., Geel, B. K., and D'Monte, B., J. Neurochem., 13, 109 (1966).

8. Rose, S. P. R., Nature, 215, 253-254 (1967).

9. Wareka, K. and Bauer, H., J. Neurochem., 14, 783-787 (1967).

10. Gaito, J. (editor), Macromolecules and Behavior, Appleton-Century-Crofts, New York, 1966.

11. Autilio, L. A., Appel, S. H., Pettis, P., and Gambetti, P., Biochem., 7, 2615-2621 (1968).

12. Austin, L., Morgan, I. G., and Bray, J. J. "The Biosynthesis of Proteins within Axons and Synaptosomes," In: Lajtha, A. (ed.), Protein Metabolism of the Nervous System, Plenum Press, New York, 271-289 (1970).

13. Whittaker, V. P., Michaelson, I. A., and Kirkland, R. J. A., Biochem J., 90, 293 (1964).

14. Jones, D. G. "On the Ultrastructure of the Synapse: The Synaptosome as a Morphological Tool," In: Bourne, G. H. (ed.), The Structure and Function of Nervous Tissue Vol. VI, Academic Press, New York, 81-124 (1972).

15. Whittaker, V. P. "The Use of Synaptosomes in the Study of Synaptic and Neural Membrane Function," In: Pappas, G. D., and Purpura, D. P. (eds.), Structure and Function of Synapses, Raven Press, New York, 87-89 (1972).

16. Shepard, G. M., The Synaptic Organization of the Brain, an Introduction, Oxford University Press, London, 1974.

17. Pittman, J. L., Thesis, East Carolina University, unpublished.

18. Cotman, C. W. "Isolation of Synaptosomal and Synaptic Plasma Membrane Fractions," In: Fleischer, S. and Packer, L. (eds.), Methods in Enzymology Vol. XXXI, Academis Press, New York, 445-452 (1974).

19. Cotman, C. W. and Tyalor, D., J. Cell Biology, 55, 697-710 (1972).