Studies on the Effects of Acetylcholine and Other Putative Neurotransmitters on *in vitro* Protein Biosynthesis in Synaptosomal Fractions of Rat Brain

by

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STUDIES ON THE EFFECTS OF ACETYLCHOLINE AND OTHER PUTATIVE NEUROTRANSMITTERS ON *IN VITRO* PROTEIN BIOSYNTHESIS IN SYNAPTOSOMAL FRACTIONS OF RAT BRAIN

A Thesis

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This study was undertaken to examine the effect of exogenous neurotransmitters, mainly ACh, on in vitro synaptosomal protein synthesis in the rat brain. Each rat brain was surgically removed, sliced and incubated with labeled leucine with and without the neurotransmitter to be tested. Following incubation, the slices were homogenized in isotonic sucrose. Synaptosomes were then isolated from the homogenates by differential centrifugation and sucrose density gradient ultracentrifugation. Synaptosomal fractions were collected, osmotically lysed, and pelleted by ultracentrifugation. Each pellet was dissolved in alkali and aliquots were analyzed for protein and radioactivity content.

Results indicated exogenous ACh had varying effects when added to brain slices from different areas of the brain. ACh and ACh with Eserine, an acetylcholine esterase inhibitor, caused stimulation of synaptic protein synthesis in some areas of the brain; inhibition of synaptic protein synthesis in others; and varying effects in still others. When Curare, a post-synaptic membrane binding agent, was added to ACh with Eserine, it did not alter the previously observed effect. This suggests that the ACh effect does not involve a post-synaptic, membrane-bound, ACh receptor protein. When Procaine, an axonal transmission blocking agent, was added with ACh with Eserine, it tended to eliminate the inhibitory responses, but also lowered the stimulatory response.

When a second neurotransmitter was added with ACh, it tended to inhibit the usual ACh effect in the cases of Noradrenalin and Serotonin; however, it did not alter the ACh effect in the case of Dopamine.

ACKNOWLEDGEMENTS

The author wishes to acknowledge the following people:

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Dr. M. V. Jackson for exemplory guidance and insights into ancient cultures, for helping me establish an early interest in science, and for keeping me alive.

Mr. A. J. Abdalla for teaching me high school chemistry.

DEDICATION

The author wishes to dedicate this thesis to all those people who have helped me to be me, who have shown me what love is, and who have helped me to learn some of the meanings and purposes of life.

To my parents and Jean Ogburn who have given me love and assistance, and have shown me my responsibilities when I needed to be shown. Thank you, I love you.

A special thanks to all my brothers and sisters--including VooDoo, Sasha, T.Z., Max and K.C., Ross and Paula, Dal and Sharlene, Ron, David and Judy, JoeJoe and Bonnie, Edwin and Marlene, Diana, Nearly Normal Norma, My Bhong Master, Bill, Jim, Monk and Linda, Niel, Gayla, Wierd Ralph, Flaky Jim, Ray and Laurie, Bill and Brenda, Paul and Lynne, Glenn and Karen, Ron and Susan, Rusty and LeVerne, Barry and Annette, Uncle Toots, Uncle Kenneth, Uncle Charlie, Budda, Garland and Marsha, Russ and Mitzie, The Rat, Miller, Bud, Schlitz, and ECU Football.

Thanks also to Justin Hayward, Mike Pender, John Lodge, Ray Thomas, Graeme Edge, Denny Laine, John, Paul, George, and Ringo, Pink Floyd, Fleetwood Mac, Alice Cooper, and Who.

The Balance

by

Graeme Edge

. . . And he thought of those he angered,
For he was not a violent man,
And he thought of those he hurt
For he was not a cruel man,
And he thought of those he frightened
For he was not an evil man,
And he understood
He understood himself
Upon this he saw that when he was of anger
or knew hurt or felt fear,
It was because he was not understanding,
And he learned, compassion.
He saw his enemies like unto himself,
And he learned love.
Then, he was answered.

And he saw magnificent perfection,
Whereon he thought of himself in balance
And he knew he was.

Just open your eyes,
And realize, the way it's always been.
Just open your mind
And you will find
The way it's always been.
Just open your heart
And that's a start.

. . . And to name this thing
Is important to some
So they give it a word
And the word is "OM"

. . . Open up the shutters on your windows
Unlock all the locks upon your doors
Brush away the cobwebs from your daydreams
No secrets come between us any more
Oh, say it's time
Only love will see you through
You know what love can do to you.

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INTRODUCTION

Little is known of the chemical mechanisms underlying learning and memory. The role of protein synthesis as a basis for these processes has been studied in the last decade. As early as 1963, Flexner, et al. demonstrated protein synthesis effects on learning and its retention. When mice were intracranially injected with puromycin, a protein synthesis inhibitor, the mice did not retain a previously trained behavior(1). This claim has been furthered by Barondes and Cohen who demonstrated puromycin memory inhibition in 1966(2); by Flexner, et al. who demonstrated in mice similar memory inhibition with acetoxycycloheximide in 1967(3). These effects have also been demonstrated in goldfish by Agranoff, et al.(4,5).

These studies are subject to criticism on the basis of the assay of memory. Learning must be inferred by an observed change in behavior in a specific task environment. Memory is tested on the basis of performance by the animal. Unfortunately, performance is affected by elements other than memory, i.e., fear, motivation, etc. Another problem is the determination of the threshold limit for the correlation between changes in the nervous system and changes in behavior. However, there is agreement that memory must involve some change at the molecular level.

One current theory is based on the premise that any learning must involve protein synthesis(5). The experiment model for this study was based on work done by Jacob and Monod in 1961, i.e., certain chemicals, known as inducters, can initiate synthesis of specific proteins(7). In the present study, the aim was to test the hypothesis that the

synthesis of brain proteins can be induced. Specifically, the intent was to examine the effect of neurotransmitters on the synthesis of synaptic membrane proteins. An inducible protein can be used as the basis of a molecular model for information storage. For example, stimulation of a neuron by a neurotransmitter may, in addition to the immediate and transient stimulatory effect, affect the synthesis of a protein which functions in synaptic transmissions, e.g., receptor protein.

One example of the many possible models of neurotransmitter action is the following: The neurotransmitter diffuses into the previously unexposed "novice" nerve cell body and into its nucleus. In the nucleus, it induces the synthesis of one or more specific proteins. These proteins can be either soluble in the cytoplasm or bound to the cell membrane. Regardless of type, these newly synthesized neuroproteins enable the neuron to "recognize" subsequent impulses and to respond to them by forming an action potential and releasing a neurotransmitter substance at the synapse with the next adjacent neuron. Thus a neuronal network or "circuit" is established.

It is not, perhaps, technically feasible at this time to test the hypothesis in a whole animal experiment involving training. Learning may involve only a few neurons (and induce only a few protein molecules). Thus the detection of an induced protein would be difficult. The experimental design therefore must overcome this problem. Rat brain slices were chosen as the experimental material because the slices could be exposed to a neurotransmitter solution such that all neurons could be affected. Thus, incubation of the slices in the presence of

neurotransmitter was considered analogous to an animal in a learning situation in that in both cases, neurons are exposed to neurotransmitter.

Even if all of the neurons respond by synthesizing specific proteins, the amount of new protein would be quite small compared to the large amount of protein normally present in brain tissue. This dictated the use of isotopically labelled amino acids in the incubation media in order to assay de novo protein synthesis(10,11,12).

Since the proteins found in synaptic membranes are clearly of importance to synaptic function, these proteins were chosen to study. It is fortunate that synaptic membranes can be isolated easily and efficiently by using sucrose gradient ultracentrifugation.

The experimental design that evolved was thus as follows: (1)Brain slices were prepared. (2)Brain slices were incubated in the presence of ^{14}C- or ^{3}H- labelled leucine, and in the presence (experimental) and absence (control) of the hypothetical inducer substance (ACh, Nor, etc.)*. (3)Synaptic membranes were isolated. (4)Samples were assayed for protein synthesis.

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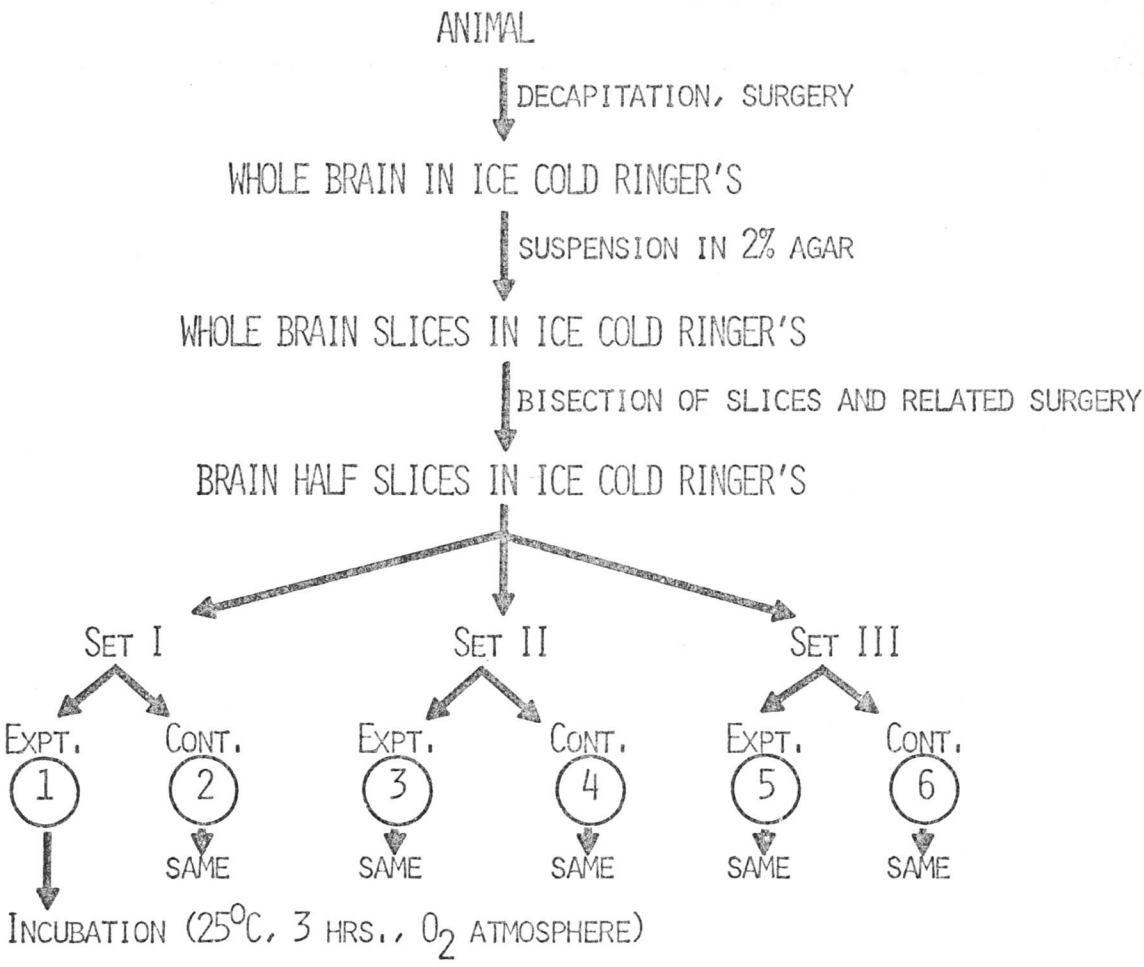
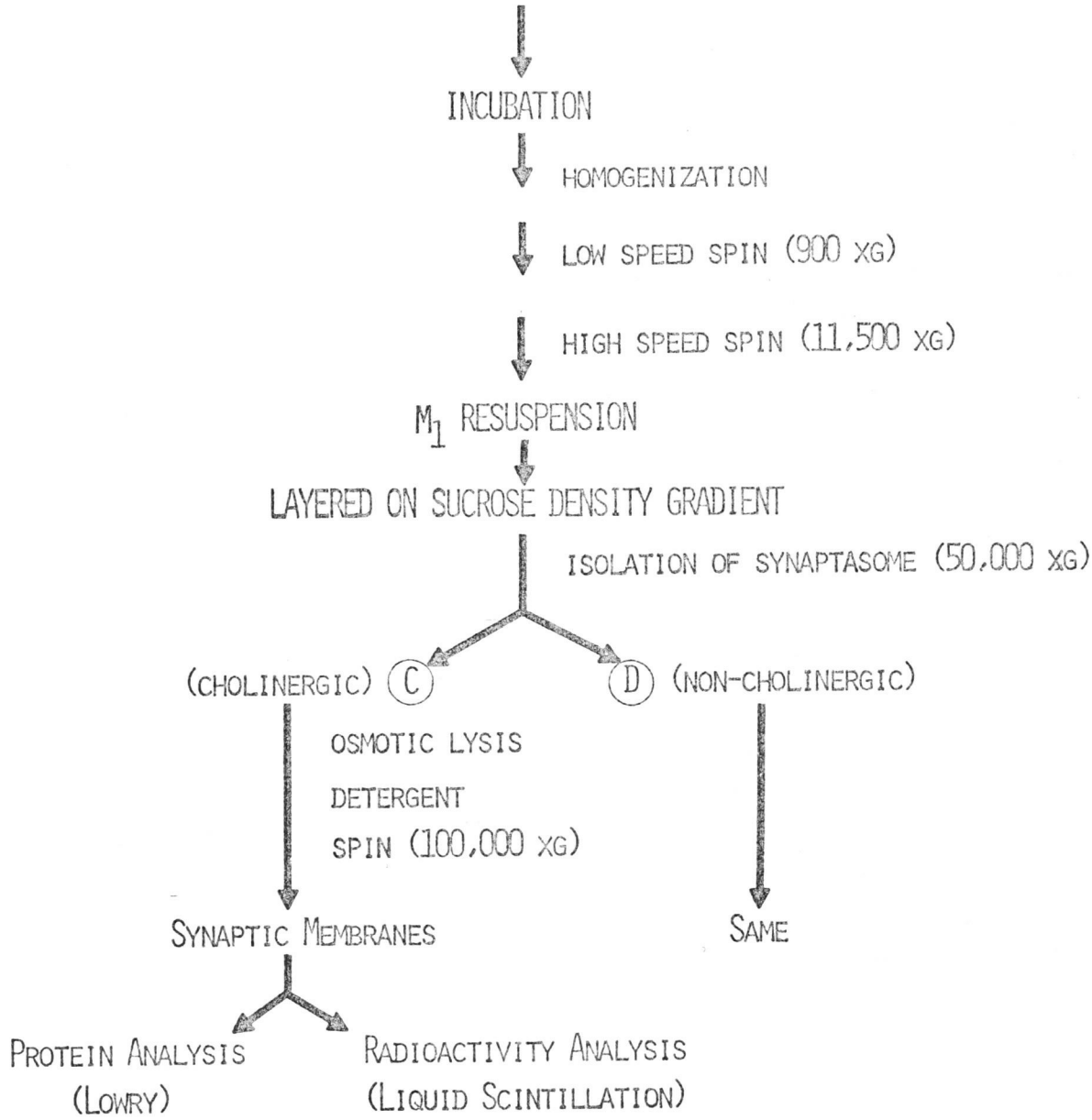
*Abbreviations used: ACh = acetylcholine, Nor = noradrenaline

Our experimental procedure was as follows:




INCUBATION
HOMOGENIZATION
LOW SPEED SPIN (900 XG)
HIGH SPEED SPIN (11,500 XG)
M_1 RESUSPENSION
LAYERED ON SUCROSE DENSITY GRADIENT
ISOLATION OF SYNAPTASOME (50,000 XG)
(CHOLINERGIC) C
D (NON-CHOLINERGIC)
OSMOTIC LYSIS
DETERGENT
SPIN (100,000 XG)
SYNAPTIC MEMBRANES
SAME
PROTEIN ANALYSIS
(LOWRY)
RADIOACTIVITY ANALYSIS
(LIQUID SCINTILLATION)

MATERIALS AND METHODS

Reagents

All reagents used in these experiments were analytical grade or better. Unless otherwise specified, these reagents were obtained from Fisher Chemical Company. All aqueous solutions were made using distilled, demineralized water with a specific resistance of approximately 15 Mohm.

A. Neural Ringer's Solution: 120 mM NaCl, 6.7 mM KCl, 1.25 mM $CaCl_2 \cdot 2H_2O$, 1.3 mM $MgCl \cdot 6H_2O$, 10.0 mM NaH_2PO_4 (monobasic), 6.0 mM glucose(8). The pH of this solution was adjusted to 7.2 with 1.0 N NaOH. It was stored at 4^oC and examined routinely for precipitation and/or bacterial growth. If either or both were present, the solution was discarded and a fresh solution prepared.

B. Acetylcholine (ACh) Solution: 10.0 mM solution in Neural Ringer's Solution (18.2 mg ACh/10.0 ml Ringer's). ACh was obtained from Sigma Chemical Company.

C. Noradrenaline (Nor) Solution: 10.0 mM solution of Nor (L-Arterenol-HCl) in Neural Ringer's Solution (20.6 mg Nor/10.0 ml Ringer's). Nor was also obtained from Sigma Chemical Company.

D. Solution of Other Drugs: 10.0 mM solution in Neural Ringer's Solution unless otherwise indicated.

E. Radioactive Isotopic Tracers:

1) L-Leucine-4,5-^3H was obtained from New England Nuclear (NET-135). It had a specific activity of 41.2 Curies/mmole in 0,01N HCl. This was diluted with Neural Ringer's to get a working solution containing 50 μCi/ml.

2) L-Leucine-^{14}C (uniformly labeled) was obtained from New England Nuclear(NEC-279). It had a specific activity of 270 millicuries/mmole in 0.01 N HCl. It was diluted with Neural Ringer's to get a working solution containing 10 μCi/ml.

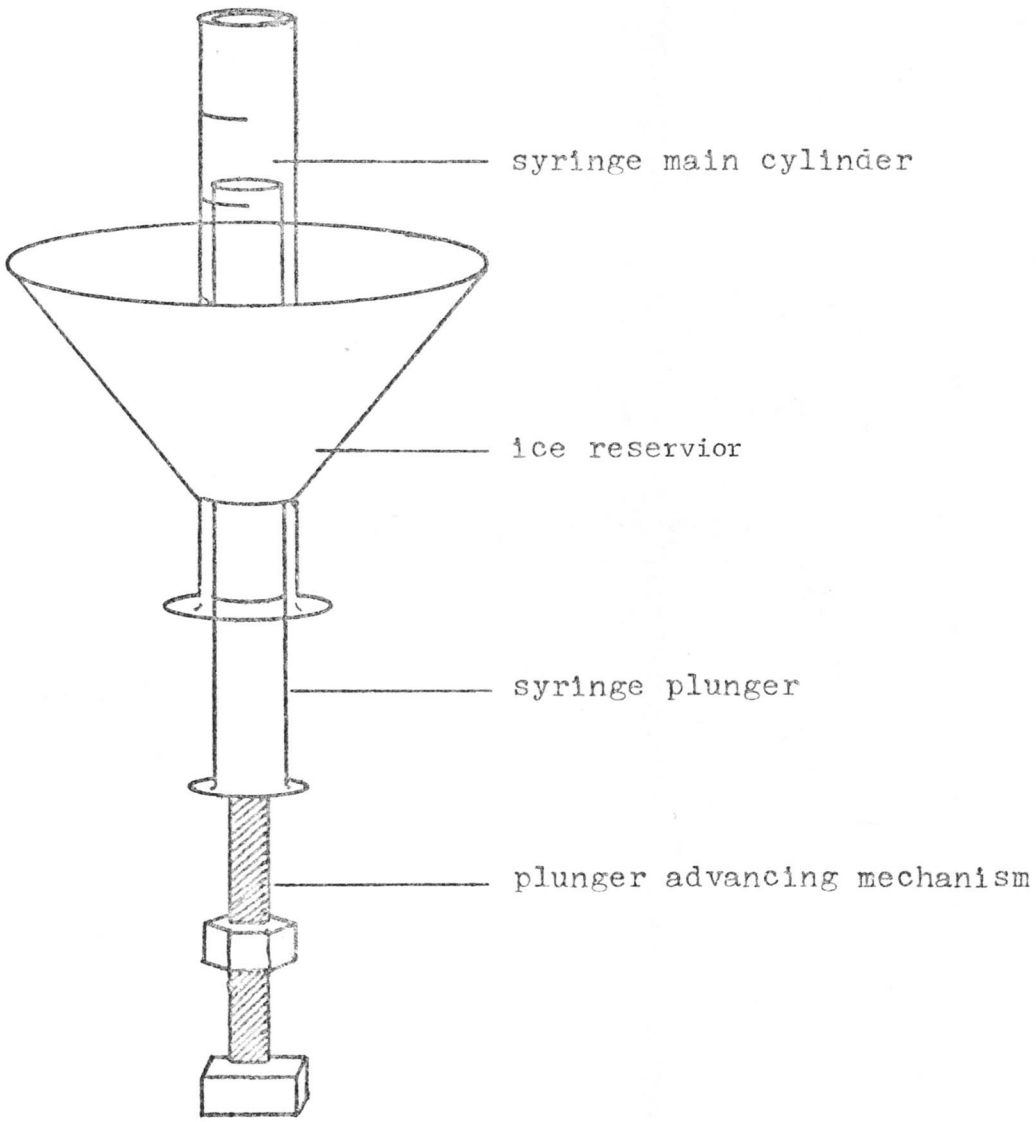
F. Scintillation Fluor "Cocktail": To 1.0 liter Toluene, Scintillation grade(Fisher Sci.Co.), was added 0.2 gm dimethyl POPOP (1,4-bis-2-(4methyl-5 phenyloxazolyl)-Benzene, Scintillation grade, obtained from Packard) and 5.0 gm PPO (2,5-Diphenyloxazole, Scintillation grade obtained from Packard). This was added to Triton X-100 (p-Iso-octyl-phenoxypolyethoxyethanol, obtained from Packard) in a ratio of 2 parts Toluene/POPOP/PPO to 1 part Triton (v/v).

Instrumentation and Apparatii

A. Slicing Aparatus: This was a 10 cc glass syringe with the main cylinder end sawed off. This was positioned in a pointed cone-shaped reservior which was filled with ice. The plunger of the syringe rested on a threaded mechanism which when rotated one-half revolution advanced the plunger upward 0.5 mm. This is shown in Figure 1.

B. Slicing Knife: The slicing knife consisted of a piece of Nichrome wire 0.07 mm thick x 1.0 mm wide x 60 mm long sharpened on one edge. This was streteched across a U-shaped stainless steel spring holder. Figure 2 shows the slicing knife.

C. 0_2 Incubation Apparatus: This was composed of a standard 0_2 cylinder, a water-filled manometer, a Ringer saturation tube, and up to six incubation test tubes. This is shown in Figure 3. The 0_2 incubation apparatus was designed with these criteria in mind: (1)0_2 cylinder-0_2 Source. (2)Manometer-to prevent a pressure buildup by using this,



Slicing Apparatus

Figure 1A

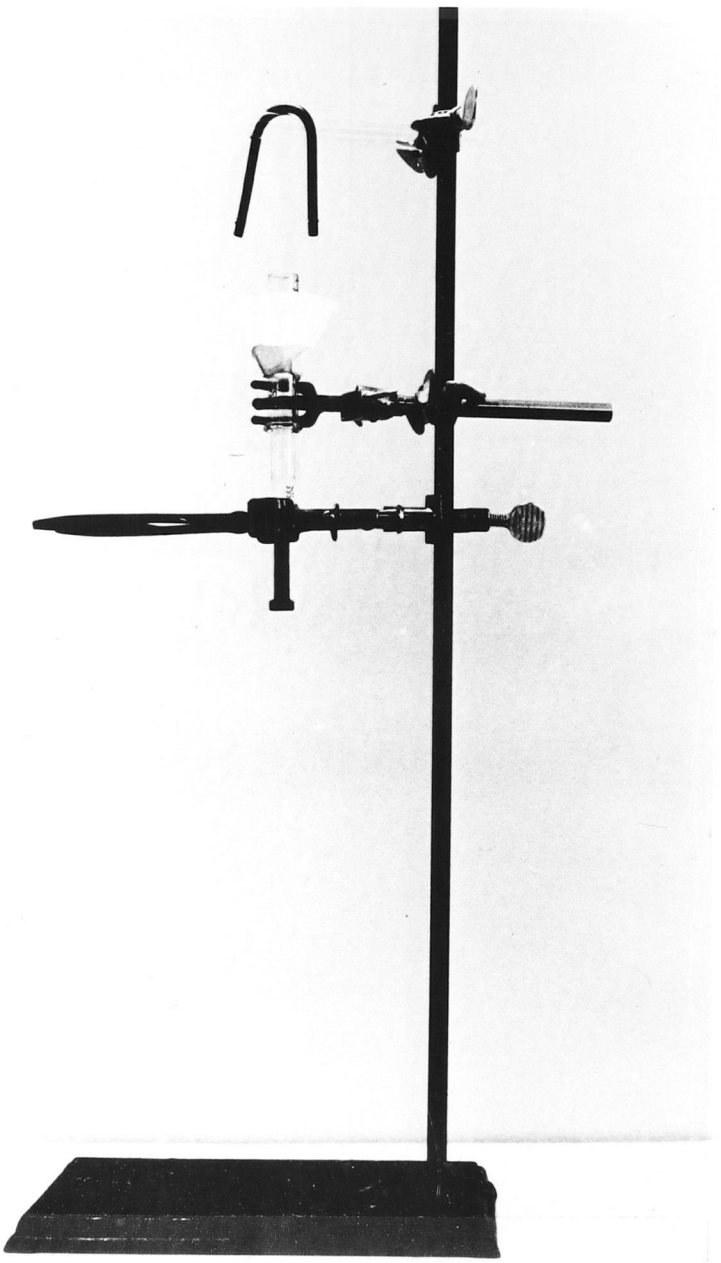


Figure 1B

any difference in 0_2 input and 0_2 exit would just displace the water in the T-shaped tube. (3)H_20 Saturation tube - this was added to decrease H_20 from the first incubation tubes dissolving in the dry 0_2 gas and thereby altering our absolute concentration. (4)Incubation tubes - this was a method of maintaining a 100% 0_2 atmosphere above the incubation media. Initially, as one tube filled with 0_2, it eventually displaced or diluted the air in each tube. This could be tested for completion by placing a flame at the outlet.

D. Homogenizers: Only Potter-Elvehjem glass/teflon homogenizers were used.

E. Centrifuges:

1) Low Speed Centrifuge: Sorvall Model #RC-3.

2) High Speed Centrifuge: Sorvall Model #RC-2B.

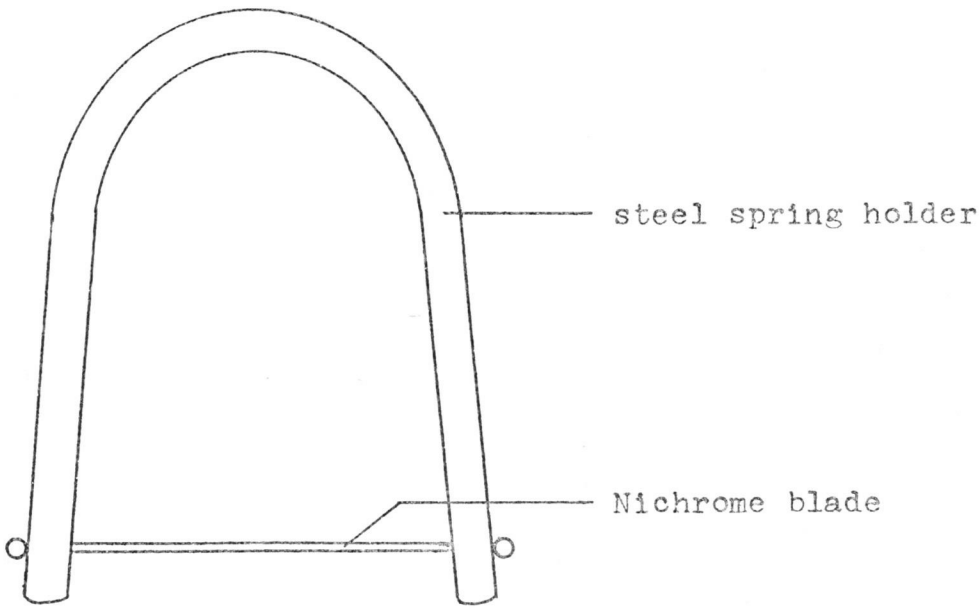
3) Ultracentrifuge: Beckman Model #L2-65B.

F. Spectrophotometer: Coleman-Hitachi Doublebeam Model 124.

G. Scintillation Counter: Hewlett-Packard Tri-Carb Scintillation Counter Model 3300 Series.

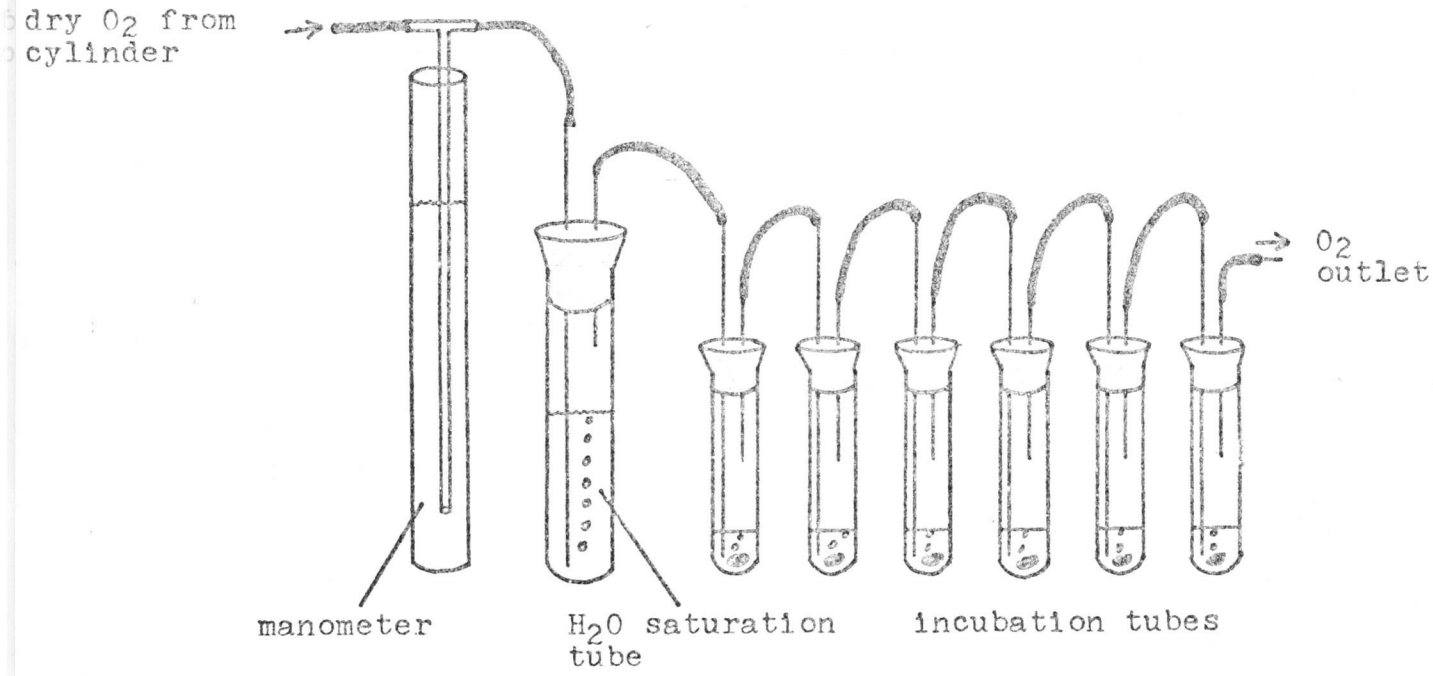
Preparation of Brain Slices for Incubation

Male albino rats, Rattus norvegicus (Sprague-Dawley strain), age 60-90 days were used. The rat was lightly anesthetized with diethyl ether, decapitated, and the brain was surgically excised within two minutes of the decapitation. It was then placed in cold neural Ringer's solution at 0^oC for approximately two minutes. Next, it was removed from the cold Ringer's and rapidly but gently blotted to remove excess liquid. It was then positioned with the anterior end of the brain upwards in the slicing syringe (see Figure 1) which was filled with a solution of



Slicing Knife

Figure 2



Oxygen Incubation Apparatus

Figure 3

2% agar agar at 35-40°C. Ice was then placed in the reservoir surrounding the syringe. This rapidly cooled the agar causing it to solidify around the brain in approximately two minutes. Slicing was accomplished by quickly pulling the slicing knife (see Figure 2) across the top of the barrell of the syringe thru the agar, advancing the plunger one-half turn, and again pulling the slicing knife across the top of the barrell of the syringe. By this method, consecutive brain slices, 0.5 mm thick, were obtained. Total time required for slicing was under three minutes. The agar-brain slices were placed into a petri dish on ice containing cold neural Ringer's. The adhering agar was gently teased away from each brain slice. Care was taken to insure the slices remained sequential. The brain slices were then divided into sets as shown in Figure 4. Each set consisted of four brain slices. This was found to be the minimum amount of brain material which gave the lowest acceptable amount of synaptic membrane material. Set 0 consisted of slices 1-4. This set was discarded for two reasons. First, due to the lobed nature of the forebrain, the slices were not continuous. Secondly, and mainly, the amount of brain material was too small. Set I always consisted of slices 5-8; Set II, slices 9-12; and Set III, slices 13-16. All slices in Sets I, II, and III were then surgically bisected into right and left hemispheres along the midline. Once again, care was taken to insure the slices remained sequential, segregated into right and left hemispheres. Additional surgery was performed on the slices in Set III. These slices passed through an area of the brain where the pituitary gland is found. Since pituitary hormones and their release factors are known to affect cellular metabolism

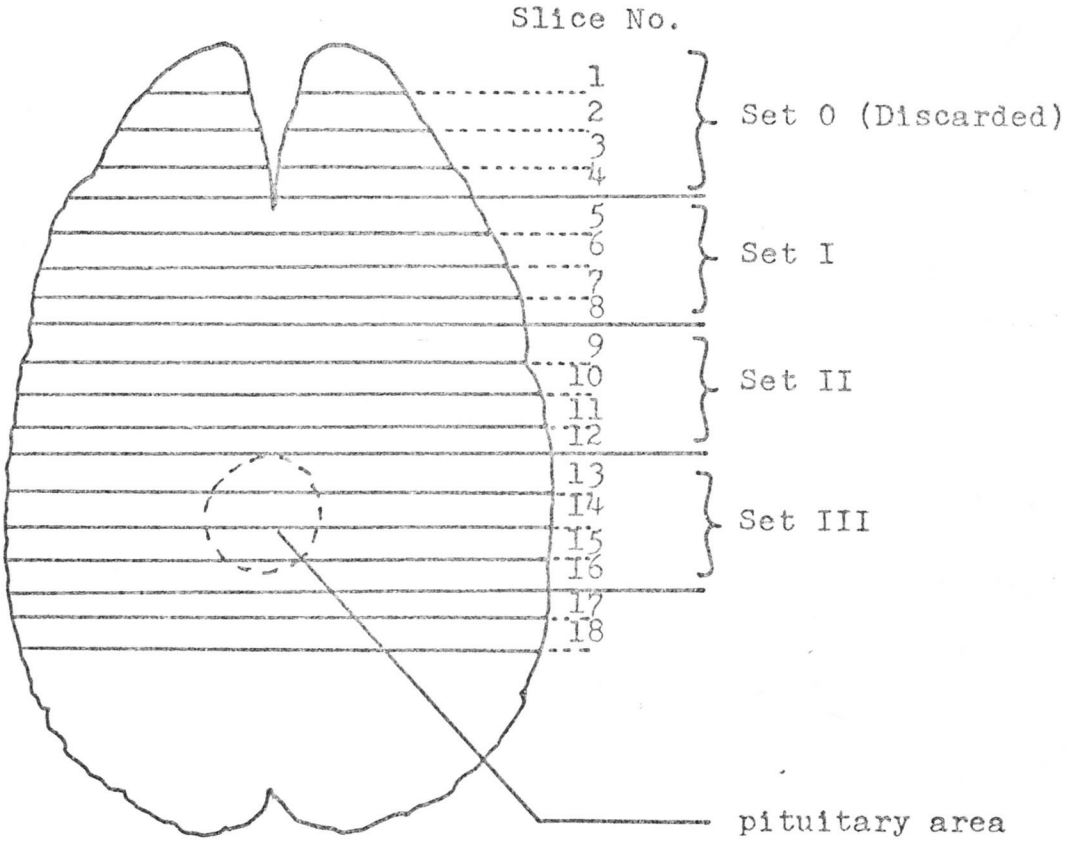


Diagram of Sliced Rat Brain

Figure 4

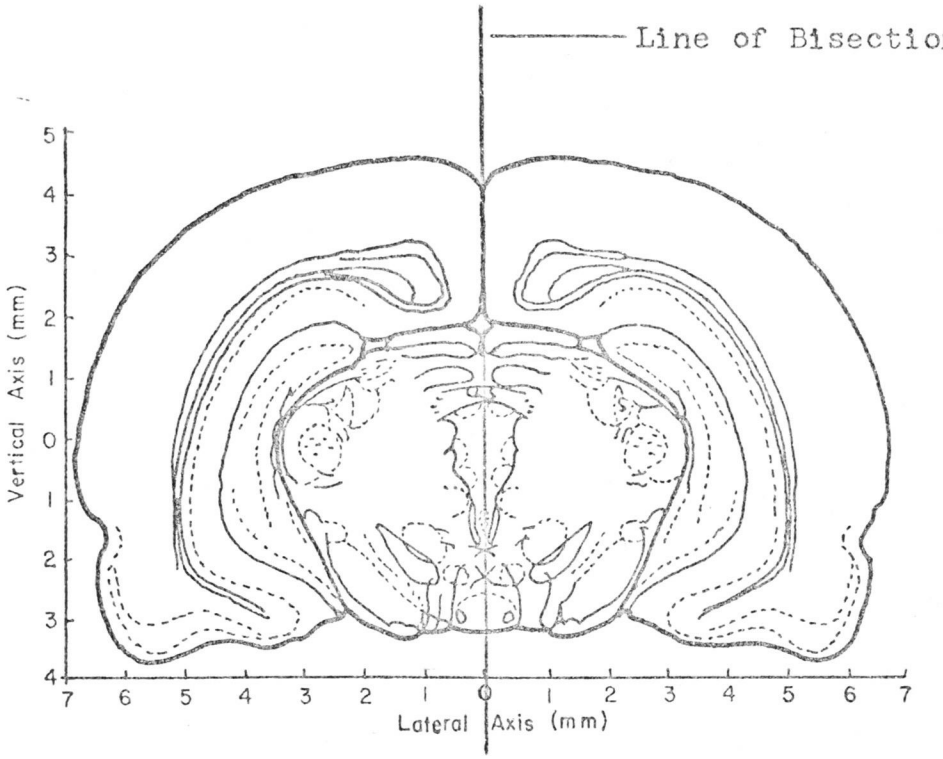
and since this would introduce another variable in our experimental system, we chose to remove this portion of the brain slice. The area removed is shown in Figure 5. In all experiments, the left hemisphere half-slices were always the experimental slices and they received the drug being tested. The right hemisphere half-slices were always the control slices.

Incubation of Brain Slices

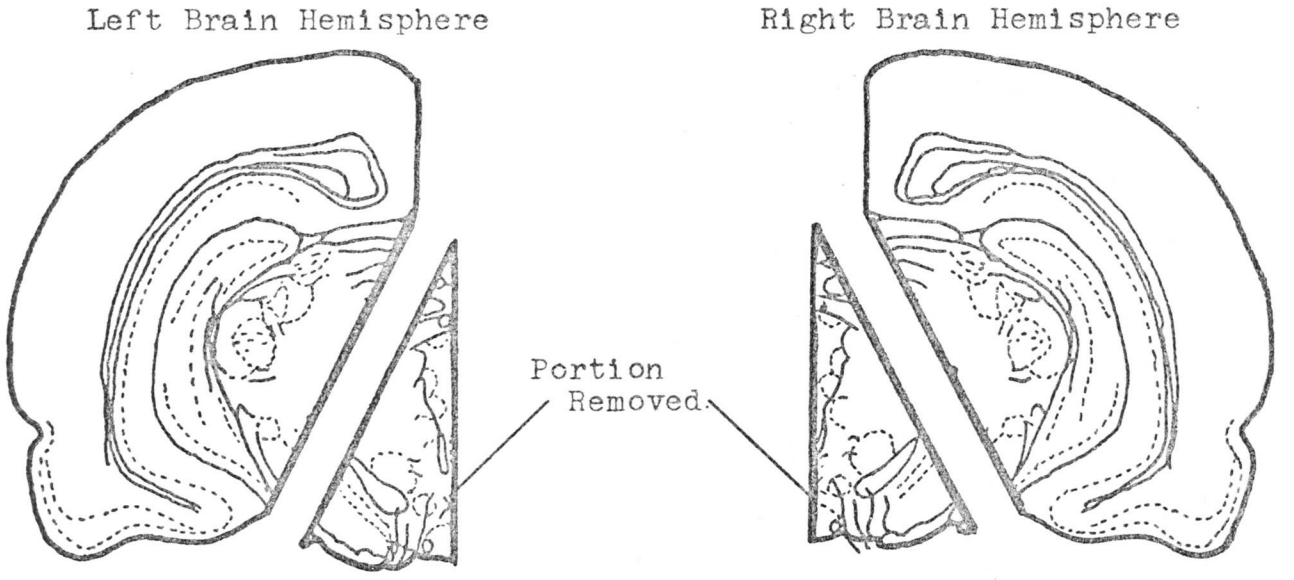
After the brain slices were divided into sets and the sets divided into right and left hemispheres, they were transferred to the incubation tubes of the incubation apparatus shown in Figure 3. These tubes were in ice and were divided into sets with an experimental and control for each of the three sets. Each set contained the following:

Experimental	Control
Four brain slices (left hemispheres)	Four brain slices (right hemispheres)
0.8 ml Neural Ringer's	0.8 ml Neural Ringer's
0.1 ml Radioactive labeled Leucine in Neural Ringer's	0.1 ml Radioactive labeled Leucine in Neural Ringer's
0.1 ml Drug dissolved in Ringer's	0.1 ml additional Ringer's

Oxygen was pumped thru these tubes while still on ice until all the air was displaced (flame test). When atmosphere above slices was O_2, the tubes were removed from the ice bath, placed in warm (30^{o}-35^{o}C) water for two minutes to raise the temperature of the incubation media. Slices were then incubated at room temperature for three hours. Orrego and Lippmann demonstrated in 1966 protein synthesis would occur at a linear rate at R.T. for three hours(9).



Typical Brain Slice from Set III



Typical Brain Slice from Set III Showing Portion Removed

Figure 5
(taken from (15))

Isolation of Synaptosomes

Following incubation, the incubation tubes were again placed on ice to prevent any additional cellular metabolism. The contents of each incubation tube was then poured into separate Petri dishes, also on ice. Approximately 15 ml of Ringer's was added to dilute and extraneous radioactivity.

The incubated slices of each tube were then homogenized in 6.0 ml sucrose, 0.32 M. Homogenization was done on ice. Synaptosomes were isolated from these homogenates according to the procedure of E. DeRobertis(10). Homogenates were then centrifuged at 900 x g for 10 minutes in the low speed centrifuge. The low speed spin removed large cellular fragments, RBC's, nuclei, and large membrane fragments. The supernatants were decanted and re-centrifuged at 11,500 x g for 20 minutes in the high speed centrifuge. The high speed spin yielded a pellet (M_1) which contained mitochondria, synaptosomes, and minute synaptic fractions. The contents of M_1 was reported by DeRobertis, et al.(10,11,12) and was confirmed in our laboratory using an electron microscope. Individual M_1 pellets were resuspended in 1.0 ml sucrose, 0.32 M, using a homogenizer, also placed in ice. The resuspensions were carefully layered onto a sucrose discontinuous density gradient consisting of layers of 5.0 ml of 1.4 M, 1.2 M, 1.0 M and 2.0 ml of 0.8 M sucrose. This gradient was prepared earlier, left at room temperature for four hours, and refrigerated at $4^{o}C$ for at least one hour prior to its use. The suspensions on the gradients were centrifuged in a swinging bucket centrifuge head (SW-27) in the ultracentrifuge at 50,000 x g for 120 minutes at $3^{o}C$. This gradient centrifugation

separated the suspension according to densities into four layers (A,B,C,D) and a pellet (E). As shown in Figure 6, DeRobertis reported that layer C contained whole, intact, cholinergic synaptasomes(12). For illustrative purposes, a typical synaptasome, also known as a "bouton," is shown in Figure 7. Fractions A,B, and E did not contain synaptosomes and were discarded.

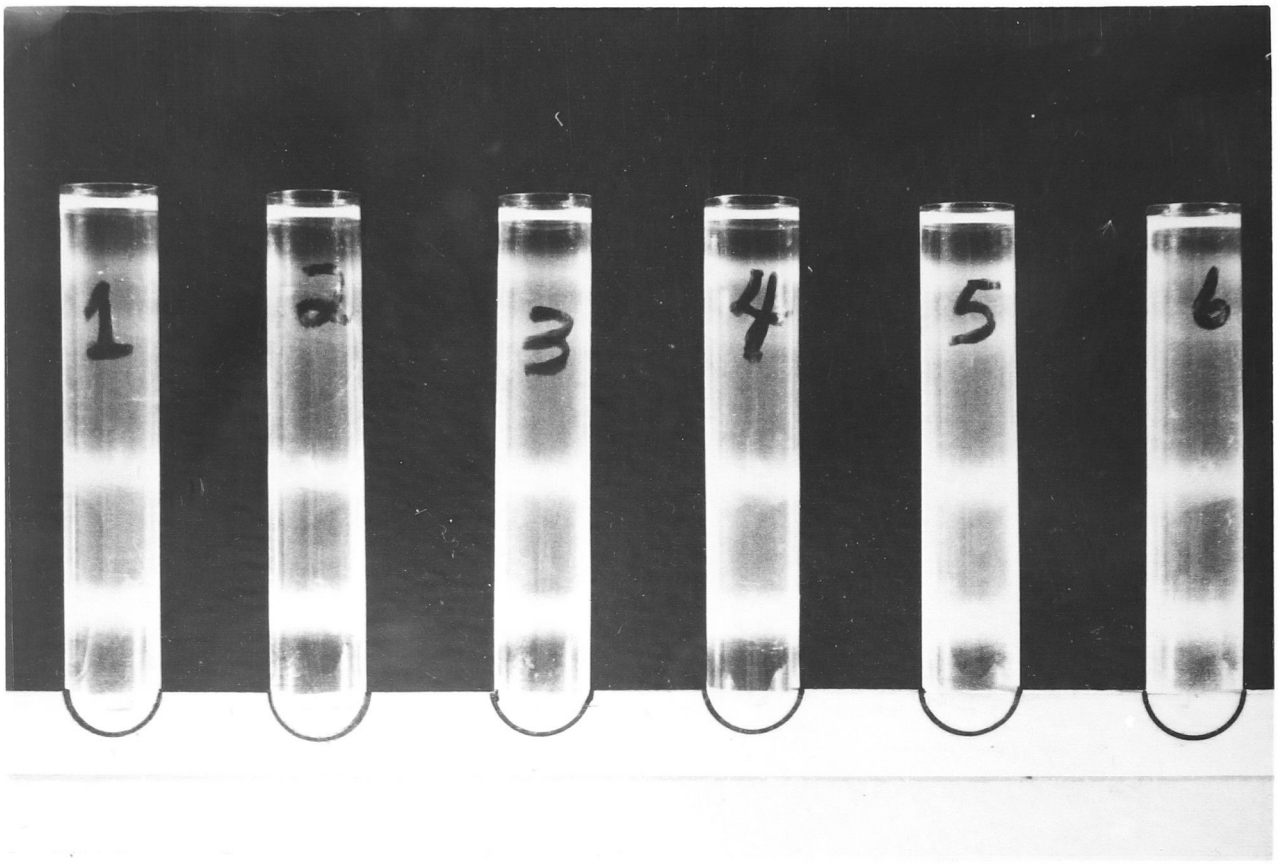
Treatment of Synaptasomes

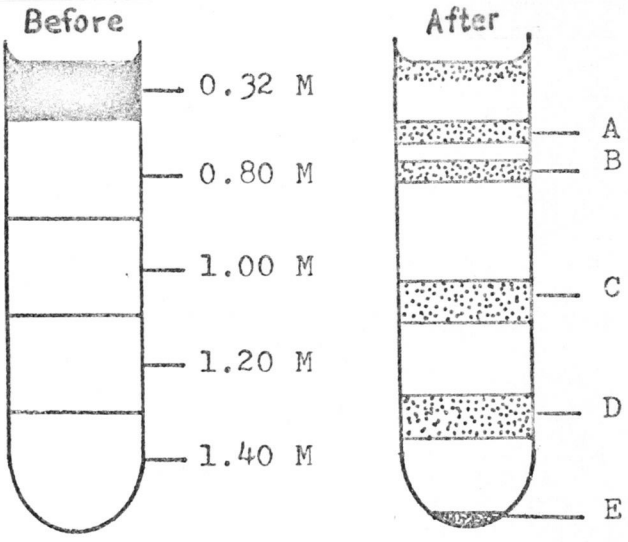
The C and D layers of each tube were collected (approximately 2 ml volume), transferred to poly-carbonate ultracentrifuge tubes, and stored overnight at $4^{o}C$. The next day, each C and D layer was osmotically lysed with 10 ml Triton X-100, 0.1% v/v, for 15 minutes at $0^{o}C$. Each was then spun in the ultracentrifuge at 100,000 x g for 30 minutes. This yielded C and D pellets of synaptic membranes. The supernatants were discarded and the pellets saved for protein analysis.

Detection of Protein Synthesis

Each C and D pellet was dissolved in 1.0 ml NH_4OH, 1.0 N. An 0.8 ml aliquot was added to scintillation vials for radioactivity measurements. To each vial was added 10.0 ml of scintillation fluor "cocktail." Samples were placed in scintillation counter and "dark-adapted" at least one hour prior to counting.

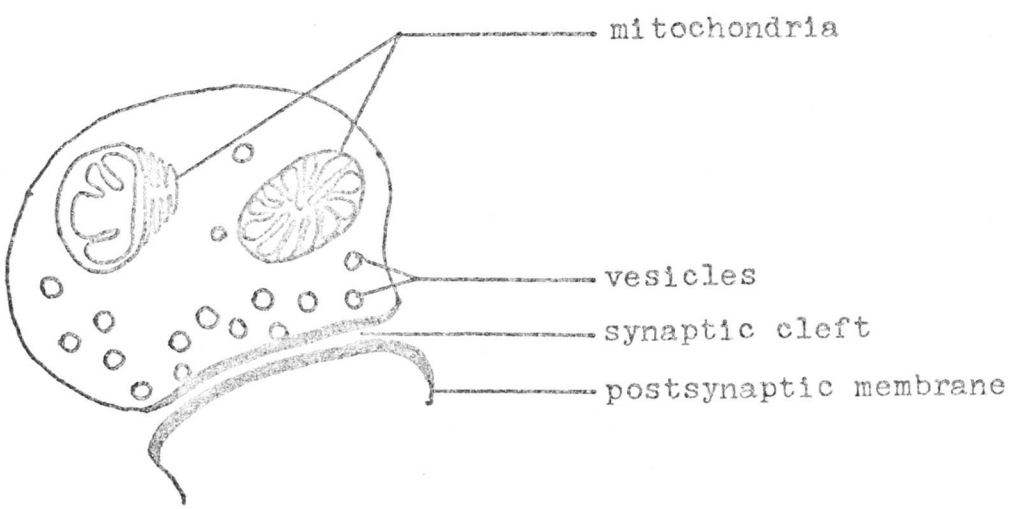
A protein analysis, by the method Lowry, et al.(13) was performed on a 0.1 ml aliquot of the dissolved C and D pellets. The standard curves were established using dilutions of a stock solution of Bovine serum albumin (BSA) containing 0.1 mg BSA/ml. See Appendix 1.





Sucrose Gradient Tube Before and After Centrifugation at 50,000 x g for 2 hours

Figure 6



Typical Synaptosome (modified from (16))

Figure 7

RESULTS

Experiments with Acetylcholine (ACh) Alone

A series of experiments was performed in which the experimental tubes contained Ringer's solution, ^{14}C-Leucine, 1 x 10^{-3}M acetylcholine, and the right half of a set of brain slices. The control tubes contained Ringer's, ^{14}C-Leucine, the left half of the same set of brain slices, but no acetylcholine. Aliquots of the final solution of dissolved synaptic membrane were assayed for radioactivity and for protein concentration. Protein was determined by a standard curve for each experiment as shown in Appendix 1. These data and the radioactivity measurements yielded an estimation of protein synthesized in units of counts per minute per milligram protein (cpm/mg). The data from one experiment is shown in Table 1. All other raw data obtained in this study is given in Appendix 2.

The results of the stimulation by ACh alone are shown in Figure 8, 9, and 10. The percent change was calculated as follows:

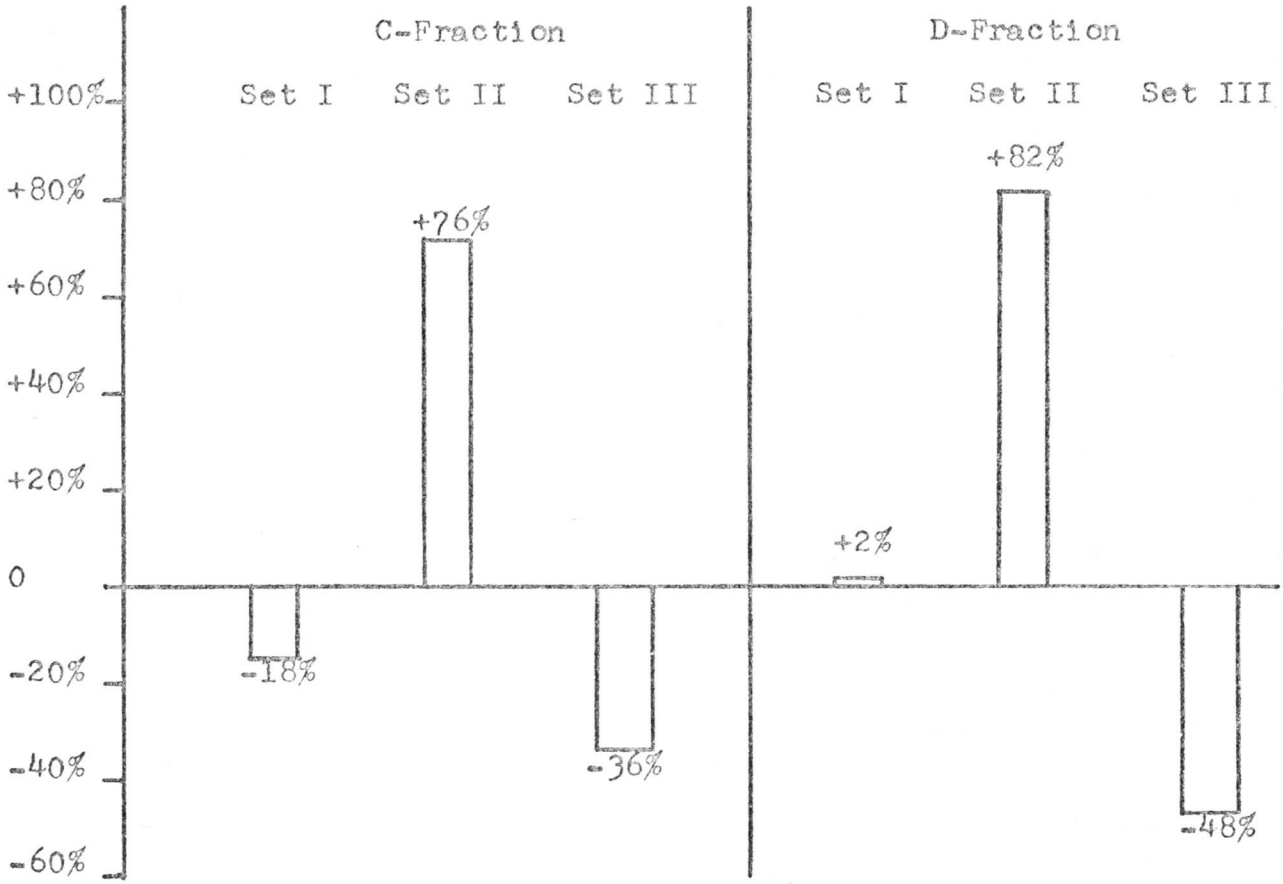
$$\% \text{ Change} = \frac{(\text{cpm/mg protein}) \text{ Experimental} - (\text{cpm/mg protein}) \text{ Control}}{(\text{cpm/mg protein}) \text{ Control}} \times 100\%$$

Table 1

Expt. 4, Ach Alone

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	+Ach	129	0.310	416	-18
	2C	-Ach	76	0.150	504	
II	3C	+Ach	115	0.273	421	+76
	4C	-Ach	74	0.310	239	
III	5C	+Ach	101	0.347	291	-36
	6C	-Ach	155	0.340	456	
I	1D	+Ach	205	0.360	569	+2
	2D	-Ach	216	0.386	560	
II	3D	+Ach	205	0.433	473	+82
	4D	-Ach	104	0.400	260	
III	5D	+Ach	143	0.470	304	-48
	6D	-Ach	194	0.330	588	

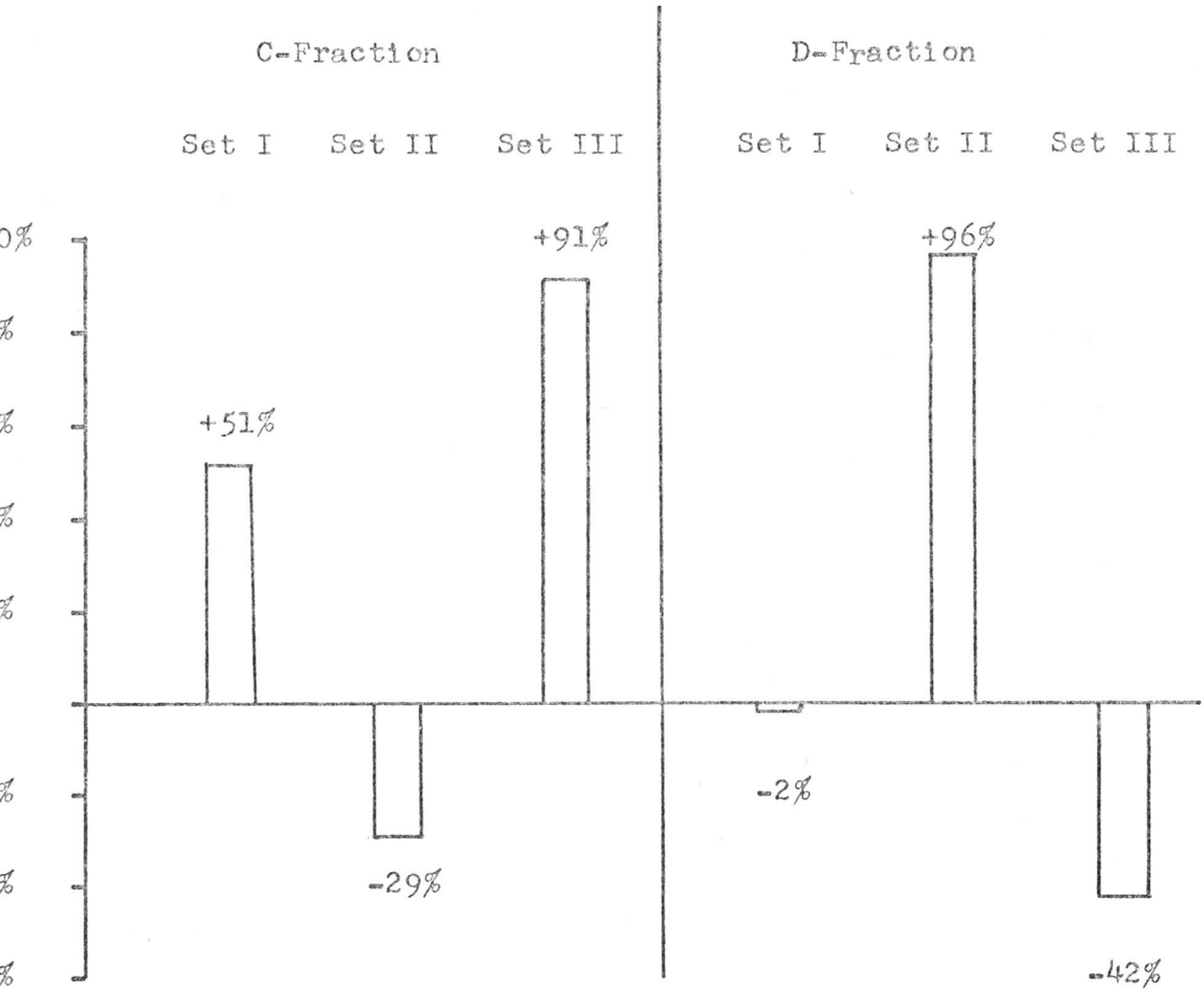
Expt. 4 - Ach Alone



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

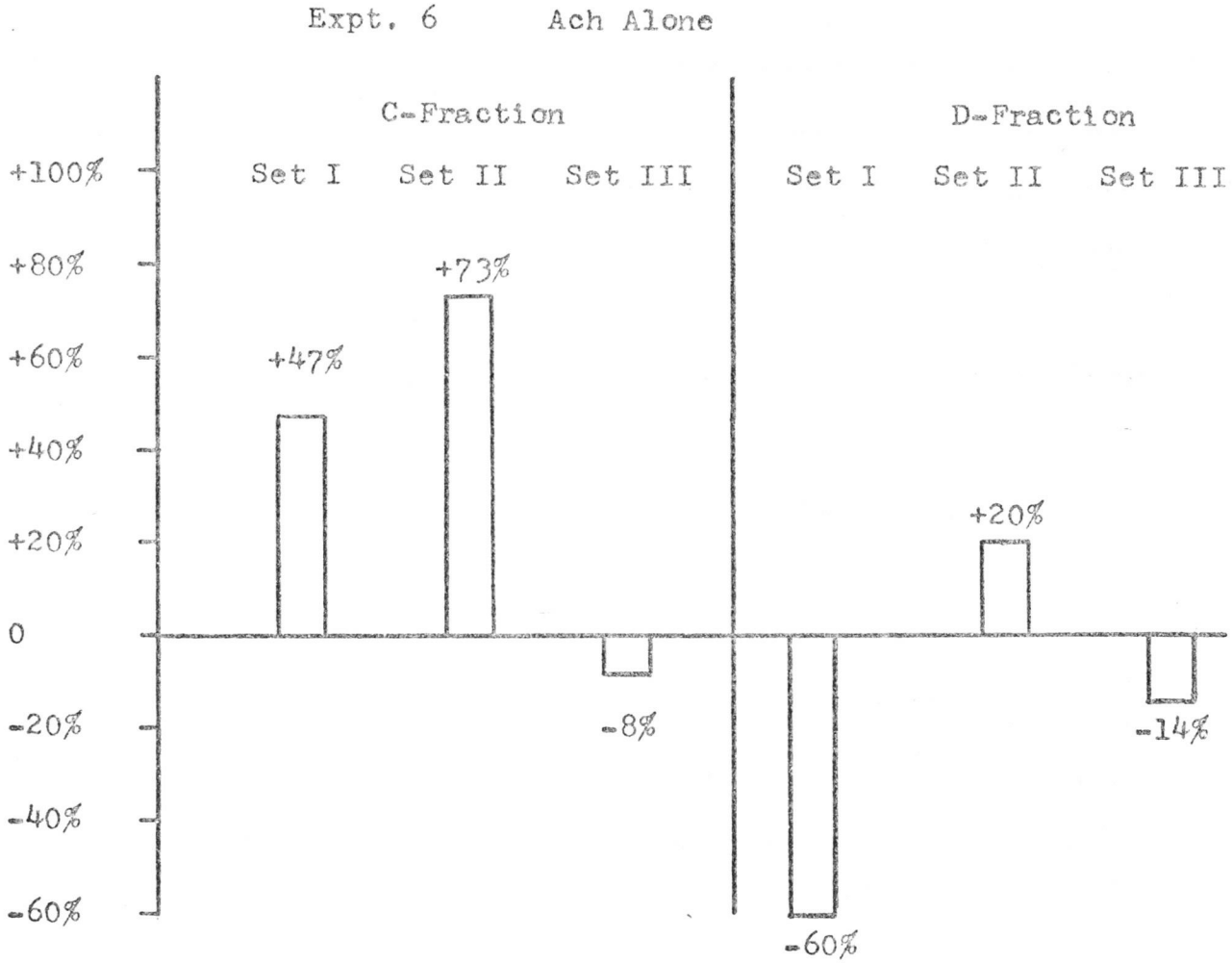
Figure 8

Expt. 5 Ach Alone



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 9



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 10

These experiments established a general pattern. Set I-D produced little or no significant change. It averaged minus 20%, with a range from plus 2% to minus 60%. Set II-D consistently demonstrated a relatively large stimulation of protein synthesis; it averaged plus 66%, with a range from plus 20% to plus 96%. Set III-D indicated protein synthesis inhibition with an average of minus 35%, with a range from minus 14% to minus 48%.

Of the C-fractions, only Set II-C exhibited a significant pattern. It showed a general synthesis stimulation with an average of plus 40%, with a range from minus 29% to plus 76%.

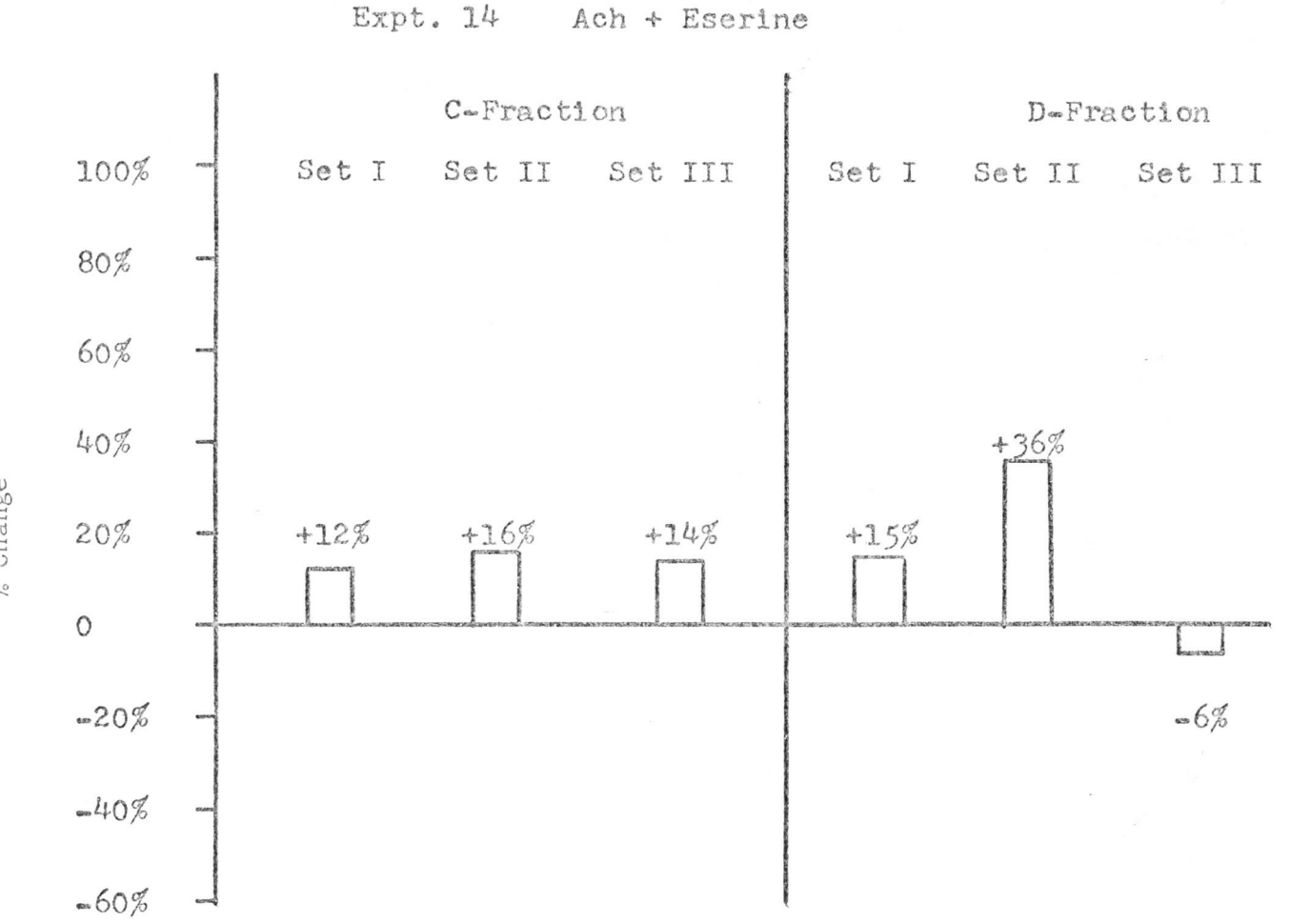
Experiments with Acetylcholine (ACh) plus Eserine (Physostigmine)

The results of a series of experiments in which the experimental tubes received ACh, Eserine (an acetylcholine esterase inhibitor), Ringer's solution, and ^{3}H-Leucine are shown in Figures 11 and 12. Experimental procedure was identical to experiments using ACh alone.

Set I-D averaged plus 33%, with a range from plus 15% to plus 51%. Set II-D shows the greatest increase with an average of 90%, with a range from plus 36% to plus 143%. Set III-D averaged minus 35%, ranging from minus 6% to minus 63%.

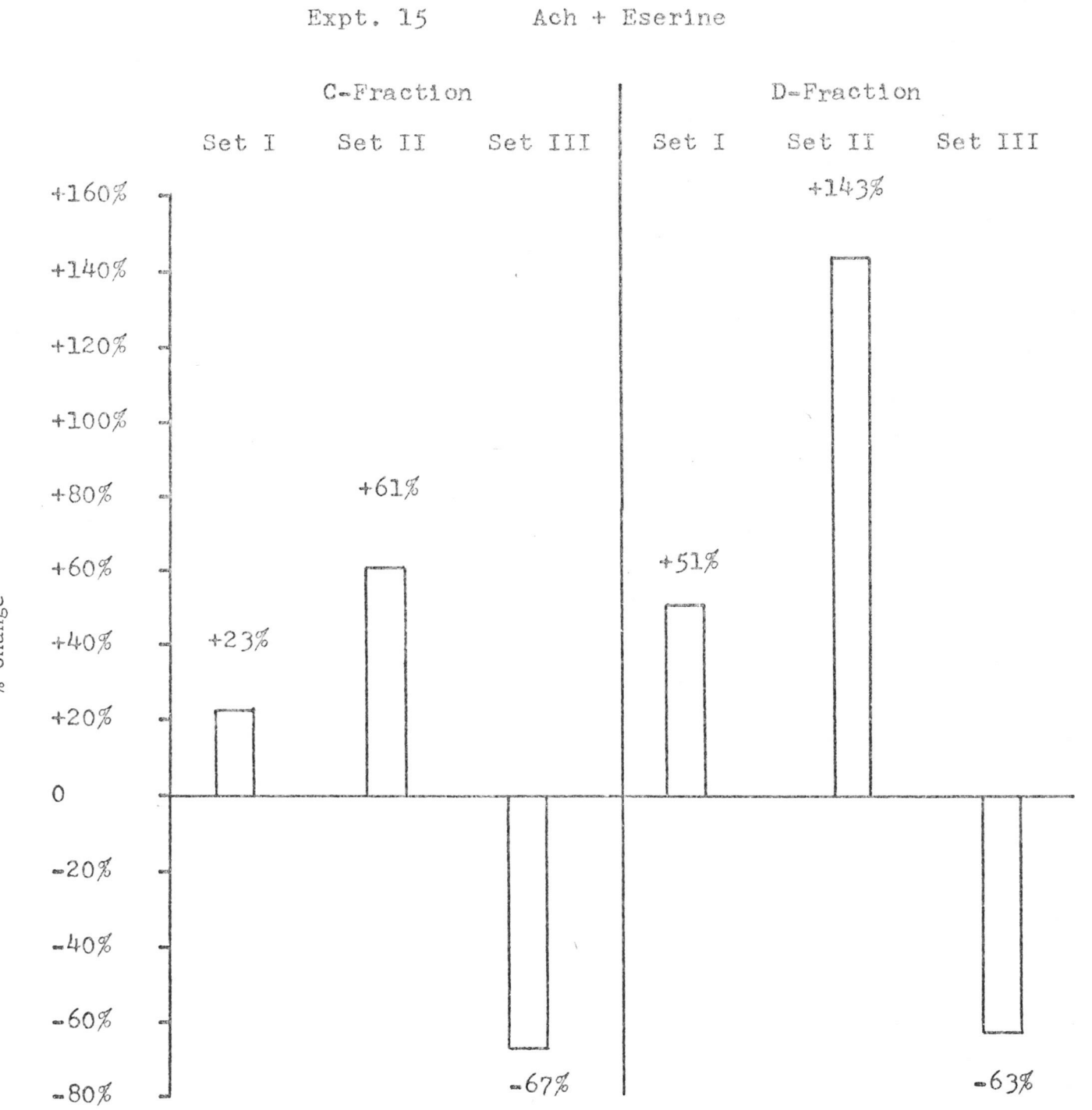
The C-Fractions exhibited positive stimulatory effects with ACh plue Eserine except in Set III. Set I-C averaged plus 18%, ranging from plus 12% to plus 23%. Set II-C averaged plus 39%, with a range from plus 16% to plus 61%. Set III-C averaged minus 27%, ranging from plus 14% to minus 67%.

The combined results of all experiments using ACh alone and ACh plue Eserine are shown in Figure 13.



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 11



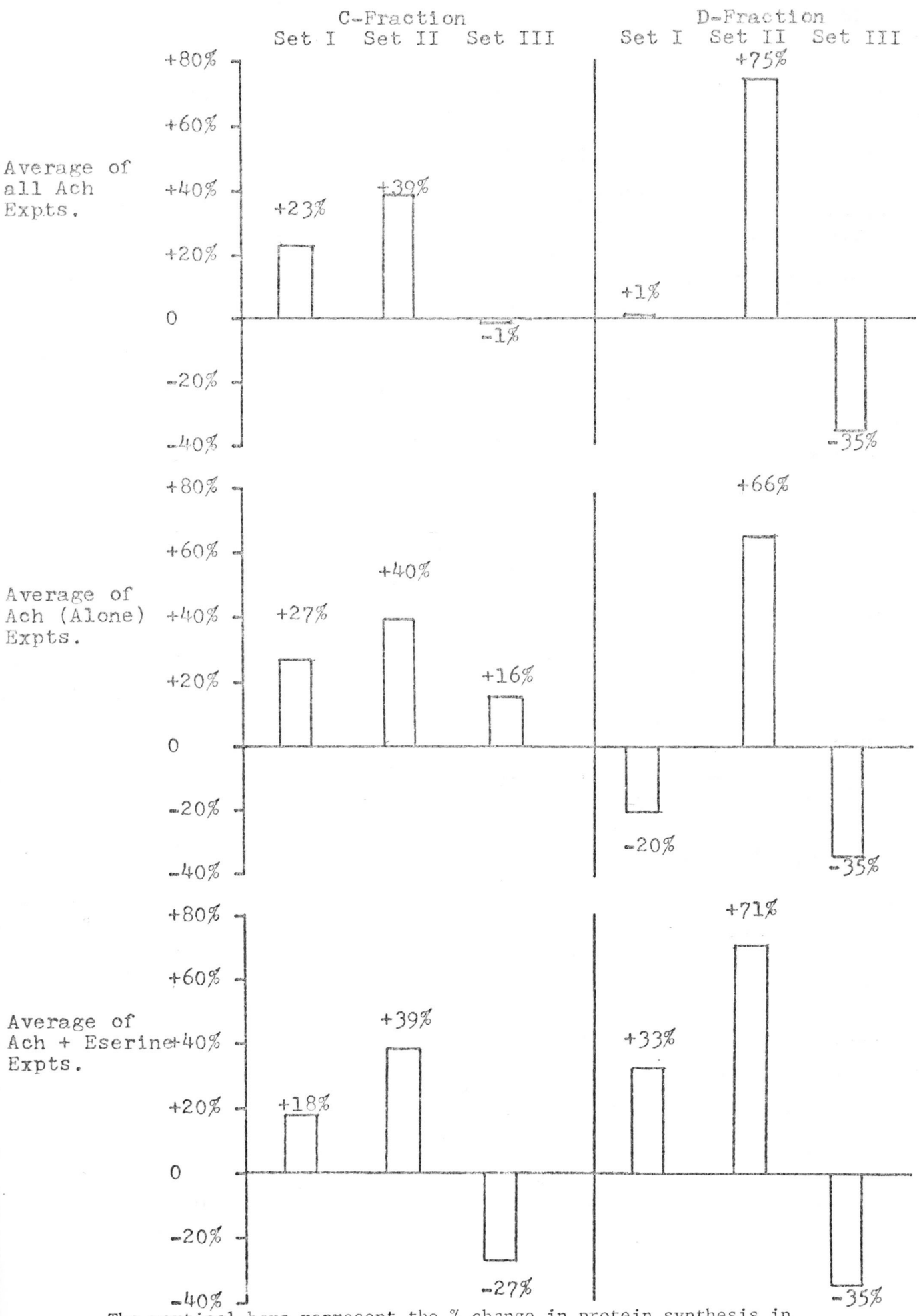
The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 12

13a. Averaged results of Figures 8, 9, 10, 11, and 12 in which ACh alone, or ACh plus Eserine were the experimental variables.

13b. Averaged results of Figures 8, 9, and 10 in which ACh alone was the experimental variable.

13c. Averaged results of Figures 11 and 12 in which ACh plus Eserine was the experimental variable.



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 13

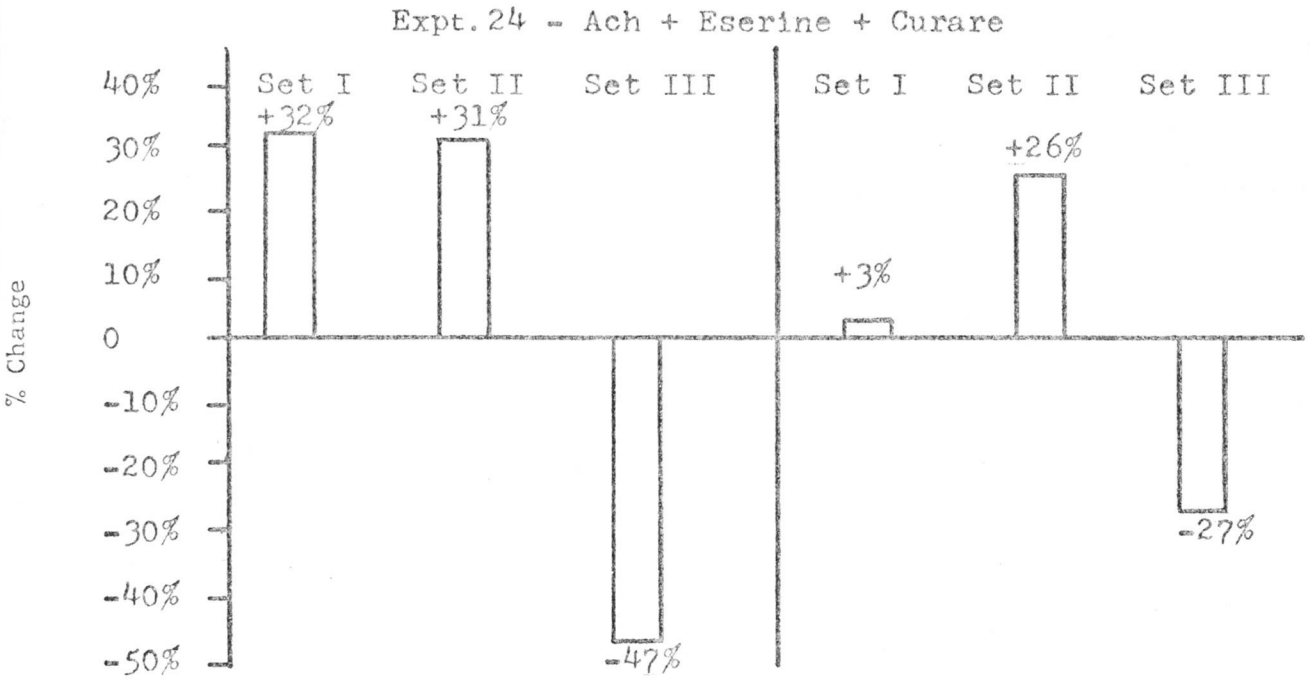
Experiments with ACh plus Eserine plus Curare

Curare (Tubocurare) was added to the experimental incubation tubes at a concentration of 10^{-4}M. ACh and Eserine were present in the usual 10^{-3}M concentrations. Curare and Eserine were added five minutes prior to the addition of exogenous ACh. The results of these experiments are shown in Figures 14 and 15. These experimental results followed the previously established pattern. Set I-D averaged minus 14%, with limits from minus 30% to plus 3%. Set II-D averaged plus 32%, with values from plus 26% to plus 37%. Set III-D averaged minus 16% ranging from minus 27% to minus 4%.

The C fractions also exhibited the same pattern. Set I-C averaged plus 8%, with limits from minus 16% to plus 32%. Set II-C had an average of plus 23%, ranging from plus 15% to plus 31%. Set III-C averaged minus 36%, ranging from minus 47% to minus 25%.

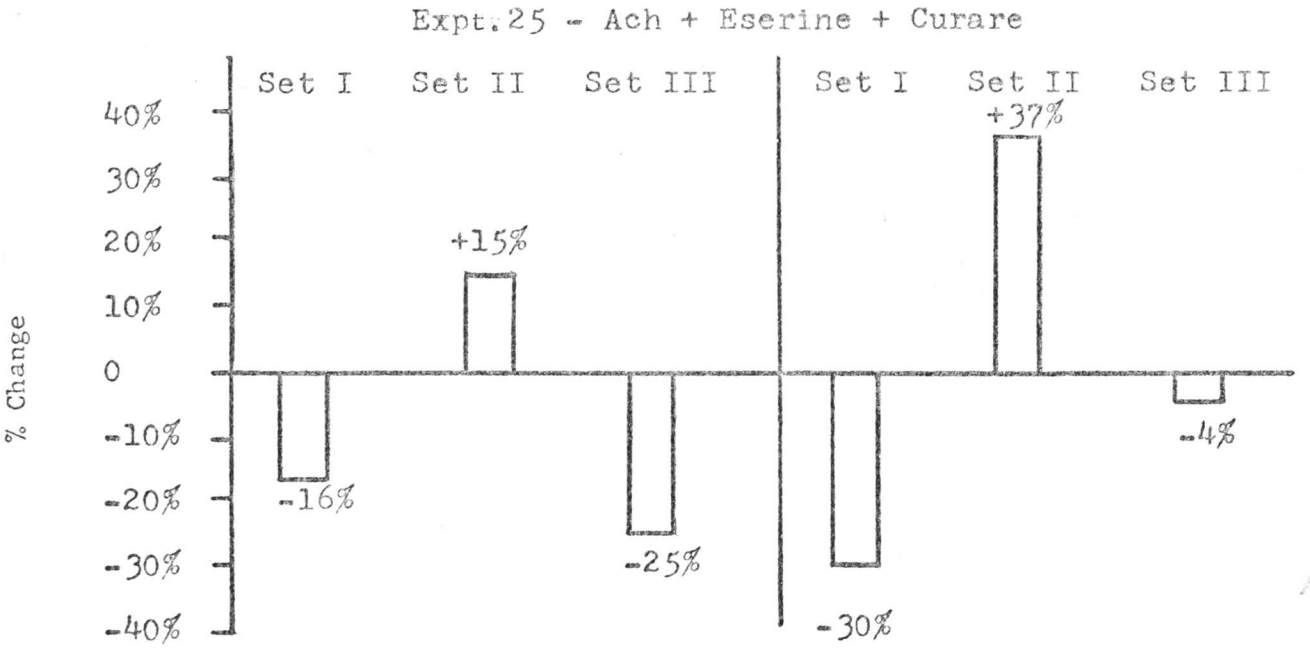
Experiments with Acetycholine plus Eserine plus Procaine $\pm$ Curare

Procaine (Novocaine) was added to the incubation media in a final concentration of 10^{-4}M. It was added in one of two combinations, either added to only the experimental sets or to both the experimental and control sets. The later was tested also with and without curare at a concentration of 10^{-4}M. Acetylcholine was added five minutes after the tissues were exposed to the above media. The results are shown in Figures 16, 17, and 18. Figure 16 shows the results of Procaine added with ACh and Eserine to the experimental tubes. Sets II-D and III-D gave the usual response, but Set II-C did not. Figure 17 shows the results when Procaine was added to both the experimental and control



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 14



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 15

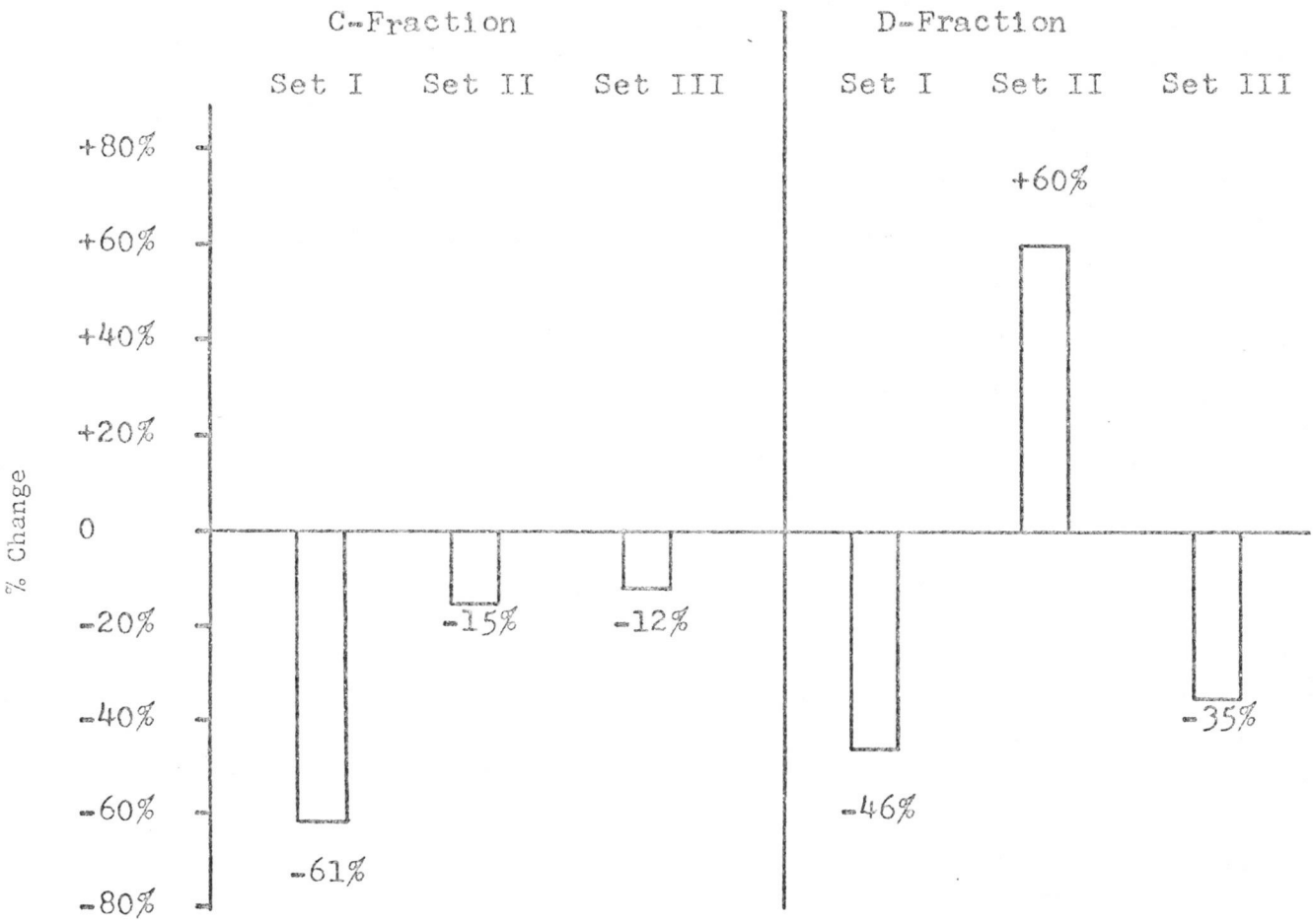
tubes with ACh and Eserine added only to the experimental tubes. Figure 18 shows results when Procaine and Curare were added to both the experimental and control tubes with ACh and Eserine added only to the experimental tubes. Figures 17 and 18 show that the inhibitory effects of ACh and Eserine are largely eliminated or reversed. Set III-D of Figure 17 showed ACh stimulation for the first time.

Other Experiments

Other, exploratory experiments were also performed. In one such experiment, the effects of ACh and Eserine were tested individually and together on brain tissue from the same animal. Tissues from Set I were exposed to 10^{-4}M ACh; tissues from Set II were exposed to 10^{-4}M Eserine; and tissues from Set III were exposed to both 10^{-4}M ACh and 10^{-4}M Eserine. The results are shown in Figure 19. These results are similar to the usual pattern, except that Set II-C was lower than usual.

In other experiments different putative neurotransmitters were tried together with ACh and Eserine. Those tested were 10^{-4}M Dopamine (Figure 20). Figure 20 shows that Dopamine does not alter the usual ACh pattern. 10^{-4}M Noradrenaline (Figure 12), and 10^{-4}M Serotonin (Figure 22). Figures 21 and 22 show that Noradrenaline and Serotonin used with ACh and Eserine tended to reduce or reverse the ACh effects; however, these were single experiments.

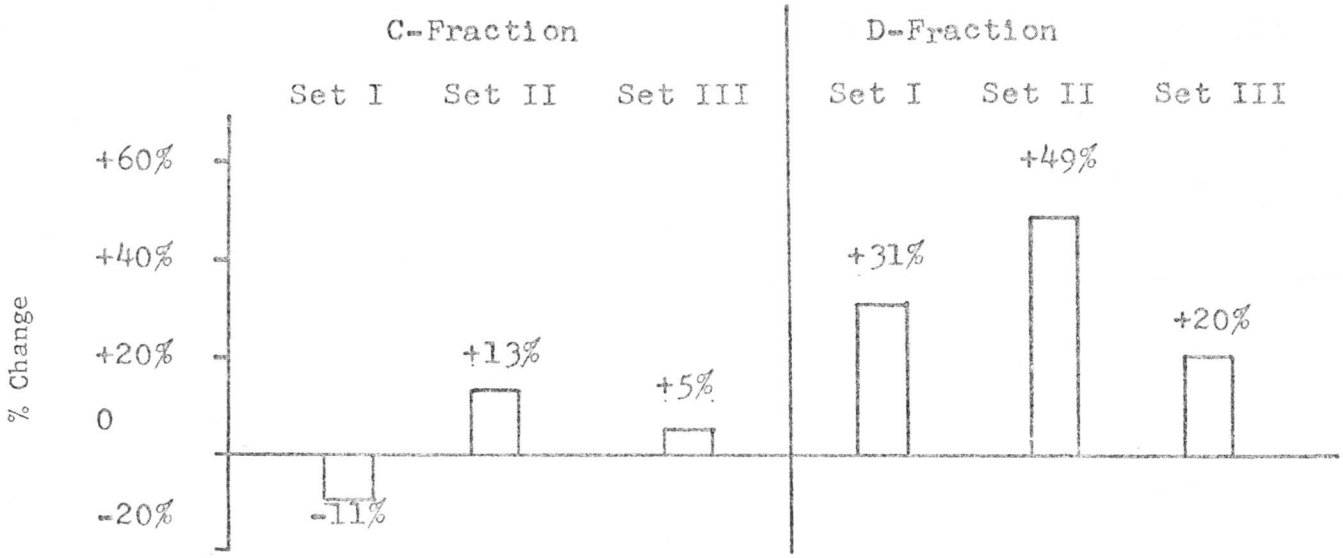
Expt. 26 - Ach + Eserine with Procaine in Experimental Tubes



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 16

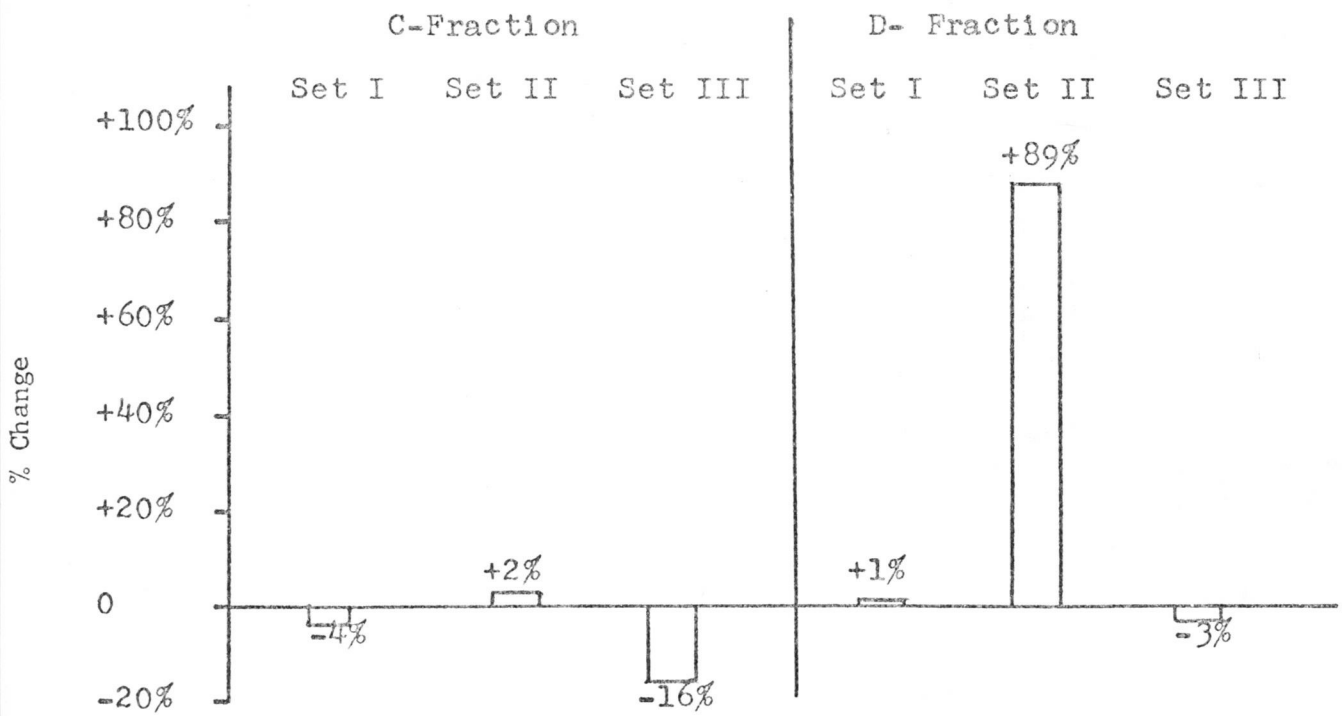
Expt. 27 - ACh + Eserine in Experimental Tubes and Procaine in Both Experimental and Control Tubes



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

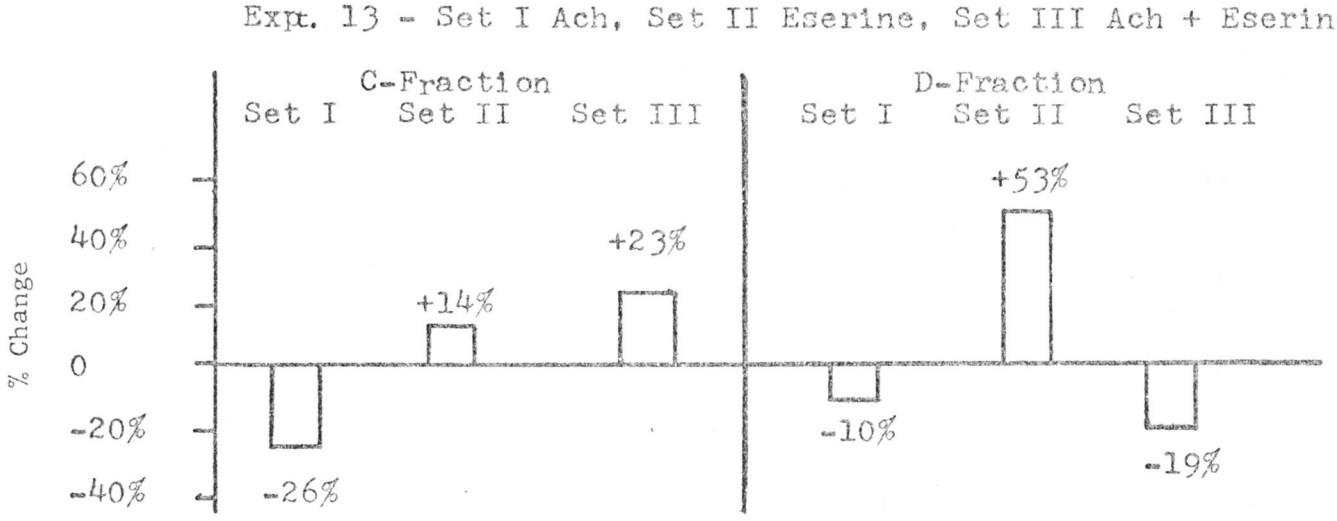
Figure 17

Expt. 28 - ACh + Eserine in Experimental Tubes and Procaine + Curare in Both Experimental and Control Tubes



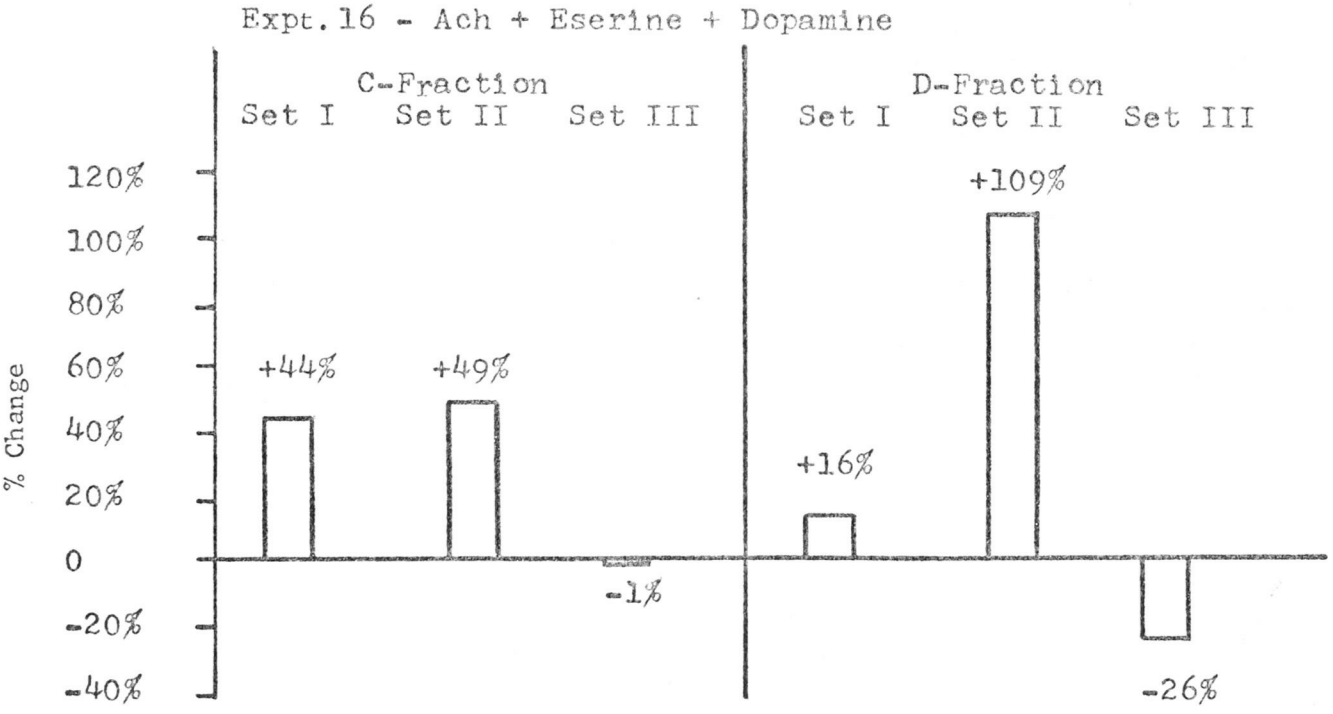
The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 18



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 19

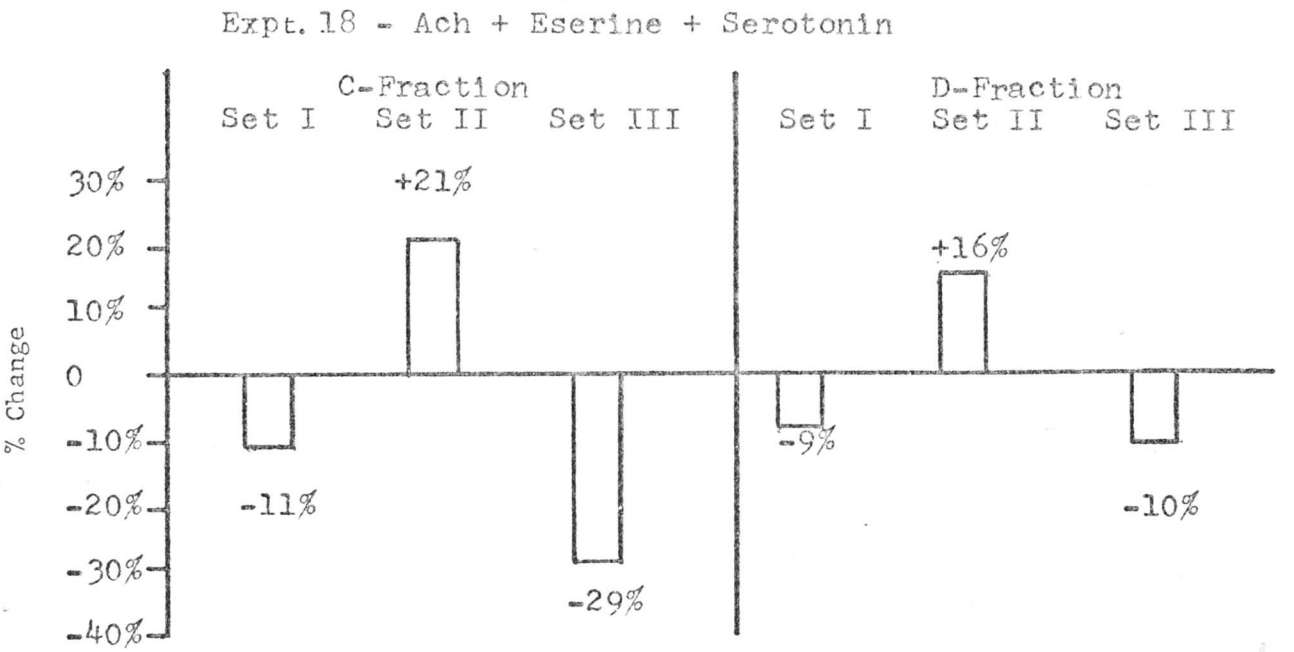


The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 20

The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 21



The vertical bars represent the % change in protein synthesis in experimental versus control as explained in the text.

Figure 22

DISCUSSION

From the experimental procedure described, one might expect one of two situations to occur:

1. All brain slices would respond in a similar manner since all the brain cells in each slice should have the same inherit capabilities. Should this occur, future studies would be greatly facilitated.

2. Brain slices from different areas of the brain might respond differently and possibly even in an opposing fashion, i.e., one set responding positively, indicating synthesis stimulation, and another set responding negatively, indicating synthesis inhibition.

Although literature search yielded no guidelines to indicate which of the two possibilities would occur, it is known that different sections of the brain vary in the number of cholinergic, adrenergic, or other synaptic types. There is no clear reason, however, to suspect the neurophysiological action of these neurotransmitters would parallel their effects on protein synthesis. The effects of acetylcholine on protein synthesis yielded an overall pattern. Set II-C demonstrated a stimulation of protein synthesis averaging plus 39%. Set II-D showed a relatively large stimulation of synthesis averaging plus 71%. Set III-D exhibited protein synthesis inhibition by averaging minus 35%. The other sets, I-C, I-D, and III-C were much more variable and showed no noticeable pattern.

This pattern was quite similar in all five experiments using either ACh alone or ACh with Eserine. In other experiments where other factors were varied, the pattern still was the same. Set II-D was largely positive, Set II-C was positive but of lower magnitude, and

Set III-C was negative.

Eserine is an acetylcholine esterase inhibitor. It potentiates the effects of ACh by preventing the hydrolysis of ACh and thus allowing for increased concentrations of ACh. The response of each set was greater when Eserine was present, probably due to higher concentrations of ACh. Generally though, the more positive II-D tended to be, the more negative III-D tended to be.

In experiment 13, (Figure 19), the experimental design was different but the same pattern existed. With experiment 16 (Figure 20), ACh and Eserine + Dopamine, the pattern was still observed. And with experiments 24 and 25 (Figures 14 and 15 respectively) using ACh + Eserine + Curare, the same pattern was present. Curare is known to bind with acetylcholine receptor protein on the post-synaptic membrane, thus minimizing interneuronal impulses produced by synaptic acetylcholine stimulation. Since the same general pattern remained unaltered with or without Curare, it suggests ACh membrane-bound receptor protein does not appear to be involved in the ACh effect upon this system. Perhaps a soluble ACh receptor protein exists in the cytoplasm of the neurons, much like the steroid receptors in chick oviduct and other target tissues(14).

Therefore, a total of nine experiments yielded the same pattern. Average values for all nine experiments are given in Table 2.

Another supporting experiment, not previously mentioned, dealt with the left hemisphere versus the right hemisphere. The sets of half-slices received incubation treatment without any added neurotransmitter, and thus the left hemisphere "experimental" tubes were the

same as the right hemisphere "control" tubes. The difference in six comparisons averaged less than ten percent.

One possible explanation for the variation in Set I-C, I-D, and III-C may be an underlying anatomical principle. It may be a secondary effect such that ACh stimulates the neuron which might in turn release noradrenaline which could then inhibit protein synthesis. The area of the brain through which the slice was taken could introduce some variation by some inhibitory feedback circuit specifically localized in that brain area.

This idea is supported by experiments 27 and 28 (Figures 17 and 18 respectively) and experiments 17 and 18 (Figures 21 and 22 respectively). Procaine and curare experiments tended to eliminate the negative (inhibitory) result of feedback circuitry by blocking all circuits. When noradrenaline and serotonin were used, the response pattern was changed. Thus the negative effects may be due to the indirect effects of other transmitters. Different slices may have varying degrees of this indirect secondary effect.

The overall conclusion is that these results suggest that further experimentation with the effects of neurotransmitters on protein synthesis is warranted. Much insight was gained by this endeavor. Future work should involve pursuing the procaine type of experiment which blocks the inhibitory cellular circuitry. Additionally, more work is required to establish the effects of other neurotransmitters and their possible synergistic or inhibitory interactions with each other. Finally, more specific protein fractions should be investigated in this experimental design.

Table 2

Averaged Results of Nine ACh Experiments

TUBE	% CHANGE
I-C	+16.5%
II-C	+34%
III-C	-6.2%

TUBE	% CHANGE
I-D	-1.7%
II-D	+66.8%
III-D	-27.6%

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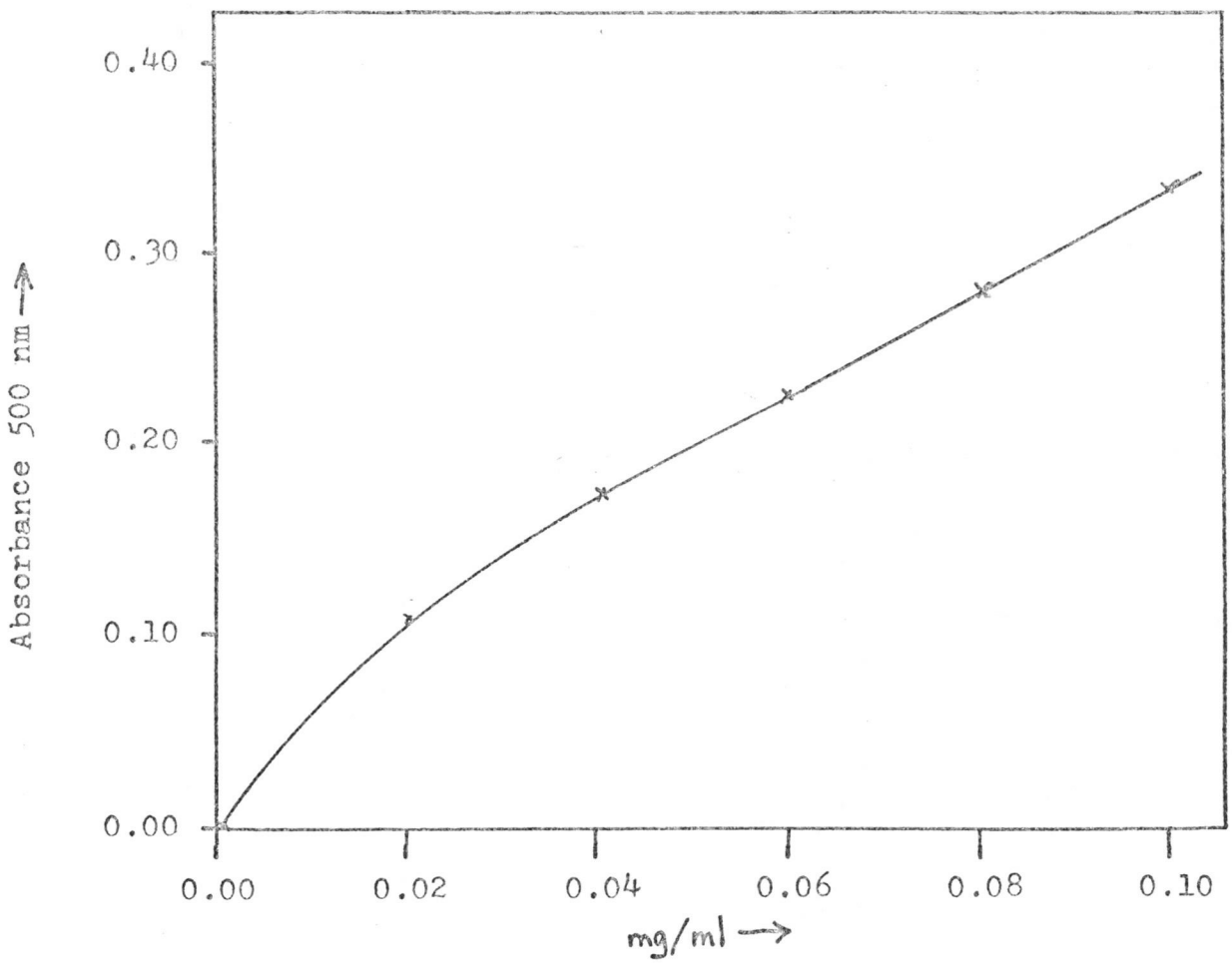
Standard Curve for Protein Determination



Appendix 1

Expt. 4, Ach Alone

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	+Ach	129	0.310	416	-18
	2C	-	76	0.150	504	
II	3C	+Ach	115	0.273	421	+76
	4C	-	74	0.310	239	
III	5C	+Ach	101	0.347	291	-36
	6C	-	155	0.340	456	
I	1D	+Ach	205	0.360	569	+2
	2D	-	216	0.386	560	
II	3D	+Ach	205	0.433	473	+82
	4D	-	104	0.400	260	
III	5D	+Ach	143	0.470	304	-48
	6D	-	194	0.330	588	

Expt. 5, Ach Alone

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	+Ach	80	0.066	1,212	+50
	2C	-	86	0.107	804	
II	3C	+Ach	62	0.150	413	-29
	4C	-	31	0.053	585	
III	5C	+Ach	54	0.090	600	+91
	6C	-	48	0.153	314	
I	1D	+Ach	181	0.109	1,661	-2
	2D	-	144	0.085	1,695	
II	3D	+Ach	130	0.102	1,275	+96
	4D	-	54	0.083	651	
III	5D	+Ach	70	0.138	507	-42
	6D	-	69	0.078	881	

Appendix 2

Expt. 6, Ach Alone

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
	1C	+Ach	123	0.011	1,118	+47
I	2C	-	129	0.170	759	
	3C	+Ach	220	0.458	480	+73
II	4C	-	75	0.270	278	
	5C	+Ach	54	0.153	353	-8
III	6C	-	126	0.330	382	
	1D	+Ach	239	0.310	771	-60
I	2D	-	325	0.170	1,912	
	3D	+Ach	291	0.550	529	+20
	4D	-	148	0.336	440	
	5D	+Ach	174	0.445	391	-14
III	6D	-	196	0.432	454	

Expt. 13, Set I ± Ach, Set II ± Eserine (E), Set III ± Both Ach and Eserine

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
	1C	+Ach	1,699	0.810	2,098	-26
I	2C	-	2,115	0.750	2,820	
	3C	+E	1,994	0.963	2,071	+14
II	4C	-	1,465	0.810	1,809	
	5C	+Both	1,789	0.967	1,850	+23
III	6C	-	909	0.605	1,502	
	1D	+Ach	2,568	0.816	3,147	-10
I	2D	-	3,200	0.920	3,478	
	3D	+E	2,293	0.862	2,660	+53
II	4D	-	1,713	0.984	1,741	
	5D	+Both	2,078	0.985	2,110	-19
III	6D	-	2,375	0.910	2,610	

Appendix 2 (cont.)

Expt. 14. Ach (A) plus Eserine (E).

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	A+E	2,210	0.520	4,250	+12
	2C	-	2,345	0.620	3,782	
II	3C	A+E	2,251	0.936	2,405	+16
	4C	-	1,495	0.720	2,076	
III	5C	A+E	1,489	0.916	1,626	+14
	6C	-	1,028	0.720	1,428	
I	1D	A+E	1,673	0.296	5,652	+15
	2D	-	1,968	0.400	4,920	
II	3D	A+E	1,849	0.570	3,243	+36
	4D	-	909	0.380	2,392	
III	5D	A+E	1,186	0.690	1,719	-6
	6D	-	991	0.540	1,835	

Expt. 15. Ach (A) plus Eserine (E).

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	A+E	2,270	0.480	4,729	+23
	2C	-	2,231	0.580	3,847	
II	3C	A+E	1,973	0.600	3,288	+61
	4C	-	1,104	0.540	2,044	
III	5C	A+E	804	0.580	1,386	-67
	6C	-	1,803	0.430	4,193	
I	1D	A+E	1,926	0.260	7,408	+51
	2D	-	2,014	0.410	4,912	
II	3D	A+E	1,681	0.300	5,603	+143
	4D	-	924	0.400	2,310	
III	5D	A+E	735	0.480	1,531	-63
	6D	-	1,773	0.430	4,123	

Expt. 16. Ach (A) plus Eserine (E) and Dopamine (D).

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	A+E+D	1,099	0.220	4,996	+44
	2C	-	1,108	0.320	3,463	
II	3C	A+E+D	870	0.320	2,719	+49
	4C	-	676	0.370	1,827	
III	5C	A+E+D	678	0.290	2,338	-1
	6C	-	799	0.340	2,350	
I	1D	A+E+D	1,523	0.220	6,923	+16
	2D	-	714	0.120	5,950	
II	3D	A+E+D	896	0.240	3,733	+109
	4D	-	571	0.320	1,784	
III	5D	A+E+D	731	0.320	2,284	-26
	6D	-	434	0.140	3,100	

Expt. 17. Ach (A) plus Eserine (E) and Noradrenalin (N).

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
	1C	A+E+N	2,394	0.630	3,800	+1
	2C	-	2,857	0.760	3,759	
II	3C	A+E+N	1,880	0.760	2,474	-7
	4C	-	1,591	0.600	2,652	
III	5C	A+E+N	1,793	0.720	2,490	+2
	6C	-	1,539	0.630	2,443	
I	1D	A+E+N	3,457	0.630	5,487	+1
	2D	-	2,994	0.540	5,444	
II	3D	A+E+N	2,220	0.540	4,111	+20
	4D	-	2,159	0.630	3,427	
III	5D	A+E+N	2,029	0.460	4,411	+21
	6D	-	1,820	0.500	3,640	

Expt. 18. Ach (A) plus Eserine (E) and Serotonin (S).

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	A+E+S	1,370	0.420	3,262	-11
	2C	-	1,727	0.470	3,675	
II	3C	A+E+S	1,670	0.540	3,093	+21
	4C	-	1,378	0.540	2,552	
III	5C	A+E+S	1,604	0.600	2,673	-29
	6C	-	1,761	0.470	3,747	
I	1D	A+E+S	1,938	0.560	3,461	-9
	2D	-	2,123	0.560	3,791	
II	3D	A+E+S	2,470	0.840	2,941	+16
	4D	-	1,578	0.620	2,545	
III	5D	A+E+S	1,864	0.600	3,107	-10
	6D	-	2,350	0.680	3,456	

Expt. 22. Serotonin (S).

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	S	1,190	0.400	2,975	-39
	2C	-	1,741	0.360	4,836	
II	3C	S	979	0.460	2,128	+14
	4C	-	710	0.380	1,868	
III	5C	S	825	0.400	2,063	-2
	6C	-	796	0.380	2,095	
I	1D	S	1,753	0.480	3,652	-41
	2D	-	2,491	0.400	6,228	
II	3D	S	1,423	0.620	2,295	+30
	4D	-	916	0.520	1,762	
III	5D	S	974	0.360	2,706	+33
	6D	-	933	0.460	2,028	

Expt. 24. Ach (A) added 5 min. after Eserine (E) and Curare (C).

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	A+E+C	1,316	0.140	9,400	+32
	2C	-	2,141	0.300	7,137	
II	3C	A+E+C	1,509	0.340	4,438	+31
	4C	-	1,530	0.450	3,400	
III	5C	A+E+C	1,150	0.260	4,423	-47
	6C	-	1,512	0.180	8,400	
I	1D	A+E+C	1,451	0.100	14,510	+3
	2D	-	1,965	0.140	14,036	
II	3D	A+E+C	1,402	0.260	5,392	+26
	4D	-	1,714	0.400	4,285	
III	5D	A+E+C	1,379	0.260	5,304	-27
	6D	-	1,897	0.260	7,296	

Expt. 25. Ach (A) added 5 min. after Eserine (E) and Curare (C).

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	A+E+C	1,099	0.140	7,850	-16
	2C	-	2,432	0.260	9,354	
II	3C	A+E+C	1,313	0.340	3,862	+15
	4C	-	1,681	0.500	3,362	
III	5C	A+E+C	1,169	0.480	2,435	-25
	6C	-	1,240	0.380	3,263	
I	1D	A+E+C	2,646	0.340	7,782	-30
	2D	-	3,754	0.340	11,041	
II	3D	A+E+C	2,028	0.380	5,337	+37
	4D	-	2,184	0.560	3,900	
III	5D	A+E+C	1,032	0.370	2,789	-4
	6D	-	1,516	0.520	2,915	

Expt. 26. Ach (A) added 5 min. after Eserine (E) and Procaine (P).

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	A+E+P	1,401	0.240	5,838	-61
	2C	-	2,407	0.160	15,044	
II	3C	A+E+P	1,496	0.480	3,117	-15
	4C	-	1,387	0.380	3,650	
III	5C	A+E+P	1,065	0.220	4,841	-12
	6C	-	994	0.180	5,522	
I	1D	A+E+P	2,958	0.340	8,700	-46
	2D	-	4,205	0.260	16,173	
II	3D	A+E+P	3,116	0.600	5,193	+60
	4D	-	2,603	0.800	3,254	
III	5D	A+E+P	1,972	0.400	4,930	-35
	6D	-	2,261	0.300	7,537	

Expt. 27. Ach (A) added 5 min. after Eserine (E) and Procaine (P) with Procaine in both experimental and control tubes.

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	A+E+P	2,279	0.360	6,331	-11
	2C	P	2,136	0.300	7,120	
II	3C	A+E+P	1,354	0.320	4,231	+13
	4C	P	1,273	0.340	3,744	
III	5C	A+E+P	780	0.380	2,053	+5
	6C	P	547	0.280	1,954	
I	1D	A+E+P	2,471	0.260	9,504	+31
	2D	P	2,171	0.300	7,237	
II	3D	A+E+P	1,693	0.280	6,046	+49
	4D	P	1,140	0.280	4,071	
III	5D	A+E+P	638	0.260	2,454	+20
	6D	P	409	0.200	2,045	

Expt. 28. Ach (A) added 5 min. after Eserine (E), Procaine (P) and Curare (C) with Procaine and Curare in both experimental and control tubes.

Brain Slice Set	Tube	Condition	cpm/ml	mg protein/ml	cpm/mg	% Change
I	1C	A+E+P+C	1,112	0.328	3,390	-4
	2C	P+C	1,205	0.340	3,544	
II	3C	A+E+P+C	903	0.330	2,736	+2
	4C	P+C	994	0.372	2,672	
III	5C	A+E+P+C	645	0.416	1,550	-16
	6C	P+C	517	0.280	1,846	
I	1D	A+E+P+C	1,477	0.328	4,503	+1
	2D	P+C	1,512	0.340	4,447	
II	3D	A+E+P+C	1,063	0.308	3,451	+89
	4D	P+C	1,048	0.574	1,826	
III	5D	A+E+P+C	588	0.348	1,690	-3
	6D	P+C	562	0.324	1,735	