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CHOLINESTERASE STUDIES ON THREE ISOLATED CHOLINERGIC SYSTEMS

by
Lloyd R. Yonce

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CHOLINESTERASE STUDIES ON THREE ISOLATED CHOLINERGIC SYSTEMS

by Lloyd R. ~~Robert~~ Yonce

This study was instituted to determine the effect of a cholinesterase (Cutter Laboratory) on three cholinergic neuro-effector systems, the vago-cardiac system of Chelydra serpentina, the chorda tympani-submaxillary gland system of the dog, and the superior cervical ganglion of the cat.

The turtle auricle was excised with the vagus nerve intact and placed in a glass receptacle containing a physiological solution. Cholinesterase, placed in the ambient solution, decreased the inotropic and chronotropic inhibition if repetitive shock stimuli were applied to the vagus nerve. According to Langmuir's theory of heterogeneous reactions, the amount of inhibition would be a function of the area available to the liberated acetylcholine and of the concentration of the acetylcholine. The concentration of the acetylcholine would be constant if the stimulus to the vagus were constant. The area available at a constant stimulation will depend on the movement of the liberated acetylcholine. Beats of the auricle will actively force the liberated acetylcholine into larger areas. The effect of the beats was shown in four ways. 1. Information on after-discharge or related phenomena would not explain the gradual increase (10-25 seconds) of inhibition with a single shock stimulation. 2. Cholinesterase will not decrease the amount of inhibition caused by a single shock to the vagus. 3. When different ambient solutions were used, the amount of inhibition varied but the number of beats remained the same. 4. At the end of a repetitive stimulation which caused complete chronotropic inhibition, the auricle must still progress through the phase of increasing inotropic inhibition before recovering. If beats occurred during the period of stimulation after cholinesterase had been applied, the phase of increasing inhibition at the end of stimulation is eliminated.

In the dog, all blood vessels of the common carotid artery except those to the submaxillary gland and all veins to the external jugular vein except those from the submaxillary gland were ligated. Cholinesterase, injected into the common carotid artery, caused less salivary flow and blood flow from the gland when the chorda tympani was stimulated.

In the cat, the isotonic contractions of the nictitating membrane were used as a criterion of the superior cervical ganglion activity when the preganglionic nerve was stimulated. Cholinesterase, applied topically or injected into the circulation, caused a decrease in the height of contraction, a greater decrease occurring at high frequency stimulation. The contraction of the nictitating membrane appears to be a function of the rate of liberation, the rate of hydrolysis, and the rate of synthesis of acetylcholine. High frequencies caused a liberation and destruction of acetylcholine more rapid than it could be resynthesized, thus causing a decline of the contraction height. The greater cholinesterase effect at high frequencies was probably the combined action of two effects, the increased rate of hydrolysis of the liberated acetylcholine and the decreased amount of liberated acetylcholine.

The reaction of each of the three effectors to repetitive nerve stimulation closely approximated the neurohumoral concept of temporal summation.

Four general conclusions may be drawn from this study. 1. Cholinesterase decreases the reaction of an effector organ to a repetitive cholinergic nerve stimulation. 2. The delay of maximum inhibition of the auricle when the vagus is stimulated with a single shock depends on the dispersion of the liberated acetylcholine by the beat of the auricle. 3. The reaction of the nictitating membrane to preganglionic stimulation depends on the rates of liberation, hydrolysis, and synthesis of acetylcholine in the superior cervical ganglion. 4. Temporal summation of each of the neuro-effector systems is in accord with the neurohumoral concept.

ACKNOWLEDGMENTS

The author wishes to express his appreciation for the facilities provided by the Department of Physiology, University of Michigan Medical School. He also expresses his gratitude to the late Professor Robert Gesell for initiating the program and providing inspirational guidance before his death. Deep appreciation is extended to Professor Charles Brassfield for succeeding Professor Gesell as chairman of this committee and for his understanding guidance and encouragement. The author also gives special thanks to Professor Russell Woodburne for reading the manuscript, to Mr. Earl Bryant for constructing special equipment, and to Mr. Alfred Pepper for valuable technical assistance. For his wife, the author gratefully acknowledges the vast amount of time she spent correcting the manuscript.

TABLE OF CONTENTS

	Page
LIST OF ILLUSTRATIONS	iv
I. INTRODUCTION	1
II. VAGO-CARDIAC SYSTEM	4
Method	4
Results	4
Discussion	29
Summary	46
III. CHORDA TYMPANI-SUBMAXILLARY GLAND SYSTEM	51
Method	51
Results and Discussion	51
Summary	61
IV. SUPERIOR CERVICAL GANGLION	62
Method	62
Results	62
Discussion	75
Summary	89
V. TEMPORAL SUMMATION	91
APPENDIX	97
METHODS	
Vago-cardiac System	97
Chorda tympani-submaxillary Gland System	104
Superior Cervical Ganglion	106
REFERENCES	110

LIST OF ILLUSTRATIONS

Figure — Page

1. Experiment showing the effect of vagal stimulation with "pericardial" and "plasma" solutions... 7

2. Graphs from figure 1..... 9
   A. Percent inhibition plotted against time.
   B. Auricular rate plotted against time.

3. Cholinesterase effect on turtle auricle with single shock stimulation to vagus nerve..... 11

4. Cholinesterase effect on turtle auricle with multiple shock stimulation to vagus nerve..... 14

5. Graph from figure 4..... 17
   A. Percent inhibition during stimulus plotted against time.
   B. Percent inhibition during recovery plotted against time.

6. Inotropic effect of cholinesterase on the turtle auricle with multiple shocks of different strengths..... 19

7. Graphs of figure 6A (three periods)..... 22
   A. Percent inhibition during stimulation plotted against time.
   B. Percent inhibition during recovery plotted against time.

8. Graphs of figure 6B (three periods)..... 24
   A. Percent inhibition during stimulation plotted against time.
   B. Percent inhibition during recovery plotted against time.

9. Graphs of figure 6C (three periods)..... 26
   A. Percent inhibition during stimulation plotted against time.
   B. Percent inhibition during recovery plotted against time.

10. Chronotropic effect of cholinesterase on turtle auricle with multiple shock stimulation..... 28

11. Graph from figure 6B..... 35
    Computed curves versus experimental data for increase of inhibition with multiple shock stimulation.

12. Graph from figure 6B..... 45
    Logarithm of percent inhibition during recovery plotted against time.

Figure

Page

13. Graph from figure 6B..... 48
Percent inhibition per second during recovery plotted against average percent inhibition.

14. Recorded salivary and blood flow of submaxillary gland with chorda tympani stimulation, before, during, and after cholinesterase..... 54

15. Graphs of figure 14..... 57
Salivary flow plotted against time for three periods, before, during, and after cholinesterase.

16. Graph of figure 14..... 60
Blood flow plotted against time for three periods, before, during, and after cholinesterase.

17. Contractions of the nictitating membrane before, during, and after cholinesterase was applied topically to the superior cervical ganglion..... 64

18. Graph of figure 17..... 67
Maximal contraction plotted against time.

19. Contractions of the nictitating membrane before, during, and after cholinesterase was applied topically to the superior cervical ganglion..... 69

20. Graph of figure 19..... 71
Maximal contraction plotted against time.

21. Contractions of the nictitating membrane when cholinesterase was injected into the circulation of the superior cervical ganglion..... 74

22. Effect of injecting cholinesterase into the circulation of the superior cervical ganglion recording contractions of the nictitating membrane..... 77

23. Graph of figure 22..... 79
Maximal contraction plotted against time.

24. Characteristics of the contraction of the nictitating membrane with high frequency stimulation to the cervical sympathetic..... 83
A. Contraction of nictitating membrane.
B. Logarithm of height of contraction plotted against time.

25. Computed curves versus experimental data for gradual increase of the height of the contraction of the nictitating membrane..... 88

Figure

Page

26. Computed curves of temporal summation of the nictitating membrane when preganglionic sympathetic is stimulated..... 95

27. Photographs of the reversible electrode used for stimulation of the vagus nerve..... 100

28. Detailed drawing of the reversible electrode..... 102

29. Photograph of reversible electrode used for stimulating cervical sympathetic..... 109

INTRODUCTION

Since the original observations of Hunt and Traveau (1906) and Dale (1914 a) that acetylcholine, in general, would mimic the parasympathetic system, innumerable experiments have been designed and considerable literature has accumulated. Nearly all of the biological experiments were either aided or hindered by the fact that acetylcholine is unstable, especially in the presence of biological tissue.

Dale (1914 b) suggested that the acetylcholine was hydrolyzed to acetic acid and choline by an esterase in the blood. Loewi and Navratil (1926) demonstrated the presence of an esterase that destroyed the "Vagusstoff" and acetylcholine. Matthes (1930) studied an esterase in horse blood, showing the monomolecular destruction of acetylcholine and the anticholinesterase property of eserine. The esterase was first called cholinesterase by Stedman, Stedman, and Eason (1932), who also produced evidence of the specificity of the enzyme. These cholinesterases are now known to be protein enzymes of a very large molecular weight (Nachmansohm, 1948).

With the existence of cholinesterase definitely established, it became necessary in the neurohumoral theories to show that the acetylcholine could be destroyed rapidly enough to conform with the time limitation placed on biological systems by the refractory periods or short activity periods. Nachmansohm (1940), analyzing the accumulated data of a number of workers, including himself, concluded that there was sufficient cholinesterase present, localized in the end plate or the ganglion, to cause the destruction of liberated acetylcholine within the required time. Neurohumoral theories of neuromuscular and synaptic transmission quickly incorporated this supporting data.

Any theory that establishes a neurohumor as a neuro-effector

or synaptic transmitting agent must be able to encompass the temporal summation which is known to occur. In Gesell's theory of neurocellular generation, temporal summation depends on the "intensification of the existing electrochemical activity. This increase of electrochemical activity could be the resultant of several factors, all related to time: 1)speed of enzymatic destruction of acetylcholine, 2)duration of periods of destruction intervening between stimuli, and 3)duration of period of stimulation or continuance of undestroyed acetylcholine at nerve centers" (Gesell and Frey, 1950). Gesell and Frey (1950) tested this hypothesis using hyperventilation produced by carotid nerve stimulation as a criterion of temporal summation. They decreased the rate of enzymatic destruction of acetylcholine by administering anticholinesterases (eserine, X-substance, DFP, and carbon dioxide); they also varied the period of destruction intervening between stimuli by applying different frequencies of stimulation. The experimental results very closely approximated the theoretical considerations. The present investigation is an attempt to increase the rate of hydrolysis of acetylcholine by the addition of cholinesterase, thus producing a decrease in temporal summation. It is important for the experiments to assume that the cholinesterase content of the tissue is normally not so great that any increase will have no significant effect.

Most studies on cholinesterase have been limited to assays of the amount of cholinesterase in terms of the amount of acetylcholine destroyed per unit time or in terms of a biological reaction to specified amounts of acetylcholine. There have been a few exceptions to this. Fredericq (1938) applied cholinesterase in the form of blood from a snail (Helix pomata) to the turtle heart. These experiments showed there was a decrease in the summation effect of two stimuli when the snail's blood was applied. Schachter (1945)

administered cholinesterase to dogs in traumatic shock and found that the blood pressure and plasma volume were increased by this treatment. His cholinesterase was made according to the method of Stedman, Stedman, and Eason (1932). Mendel and Hawkins (1943) tested the effects of cholinesterase preparations on acetylcholine injections. They found that the cholinesterase prevented excessive salivation, intensive lacrimation, and chromodacryorrhea in dogs. More recently, Beck (1952) studied the effect of intravenous injections of "true" cholinesterase made from bovine erythrocytes. This caused no detectable reaction of the rat sciatic-gastrocnemius preparation or the phrenic-diaphragm preparation, even when the animal had been previously poisoned with anticholinesterase drugs. Beck concluded that either the cholinesterase did not reach the structures in the end plate where hydrolysis of acetylcholine normally occurs, or, if penetration did occur, the concentration of the enzyme was already so high that any increase was not detectable by pharmacological methods.

Since only a relatively few biological observations have been conducted by applying a cholinesterase preparation, it seemed advisable to obtain general information of the effect of cholinesterase on some cholinergic systems.

The purpose of this investigation is two-fold, to supply additional and supporting data to the neurohumoral mechanism of temporal summation as proposed by Gesell's theory of neurocellular generation, and to accumulate miscellaneous information from the application of cholinesterase.

The preparations used were the vago-cardiac system of Chelydra serpentina, the chorda tympani-submaxillary gland system of the dog, and the superior cervical ganglion of the cat.

VAGO-CARDIAC SYSTEM

Method

The preparation used was the excised auricle and intact vagus nerve of Chelydra serpentina. The auricle was placed in a receptacle for topical application of different solutions and the automatic beats were isotonically recorded on a kymographion. The vagus nerve was stimulated through reversible fluid-bridge electrodes connected to an electronic stimulator. Details of the method are given in the appendix.

Results

Stimuli were of two types, single shocks and multiple shocks. Single shocks consisted of a specific strength and duration, only one shock being applied. Multiple shocks were a definite number of single shocks applied at a low frequency, usually one per two seconds.

The duration of each single shock was maintained at a low value, about 0.2 milliseconds, to minimize the repetitive stimulation of the rising applied potential, followed, after the duration period, by a falling applied potential. Keeping the duration short would make the second stimulus fall within the absolute refractory period.

The strength of a stimulus needed to produce an inotropic or chronotropic inhibitory effect on the auricle when the vagus was stimulated was not the same for different turtle heart preparations. The difference may have been due to variations in dissection technique in exposing the vagus nerve. More of the sheath may have been removed on one turtle than another. Another possibility is that the normal resistance of the nerve sheath varies from one vagus to the next, depending on possible structural variations.

The inorganic salt solution used determined the amount of

inhibition obtained from a stimulation of the vagus nerve. The "plasma" solution had a pH of 7.75 and the "pericardial" solution had a pH of 8.15 as measured on a Coleman pH meter. If an auricle was placed in "pericardial" solution after isolation from the turtle, the amount of inhibition decreased with single shocks of the same strength and duration applied to the vagus until a constant level was reached (figure 1). The inhibition started at about 47 percent and decreased to 10 percent. Then placing the auricle in "plasma" solution caused the inhibition to increase from 10 percent to 50 percent where it appeared to level off and then subsequently increased to a higher level of about 65 percent. This second increase in inhibition coincided with the placing of fresh "plasma" solution on the auricle. The percent of maximum inhibition was plotted against time to show the progressive change (figure 2A).

Some auricles gave more stable reactions in "pericardial" solution and some responded better in "plasma" solution. Regardless of which one was used for cholinesterase application, the auricle was allowed to stabilize until a constant amount of inhibition could be obtained with a given stimulus to the vagus.

The initial experiments were conducted using a single shock stimulation. It was anticipated that a decrease in the amount of inhibition from the single shock would result from the application of cholinesterase. However, there was never a decrease in the amount of inhibition with the single shock stimulation (figure 3). Calculations for the experiment illustrated were typical. They show that the inhibition prior to the cholinesterase was 42 percent, during cholinesterase application was 46 percent, and after cholinesterase was 40 percent. The 4 percent increase is contrary to the original supposition. This may not be a significant change, but if it is, it may be accounted for by an item to be discussed under a

Figure 1. Experiment showing the effect of vagal stimulation with "pericardial" solution and "plasma" solution. "Pericardial" solution was applied from 0 minutes to 84 minutes. "Plasma" solution was applied from 84 minutes to 170 minutes. The "plasma" solution was changed for fresh solution at 135 minutes. Strength of stimulation was 10 volts. Duration of stimulus was 0.2 millisecond.

FIG. 1

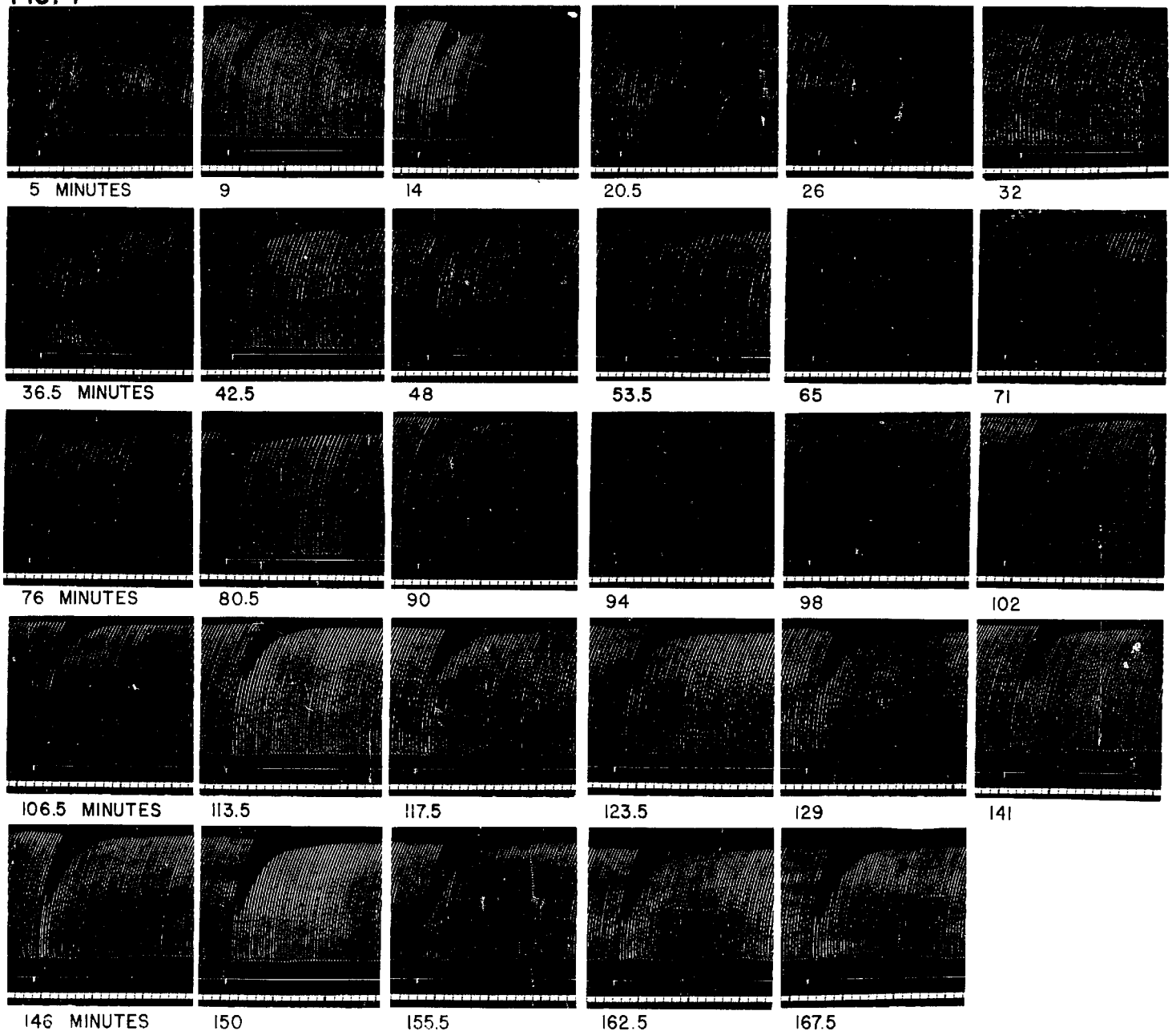
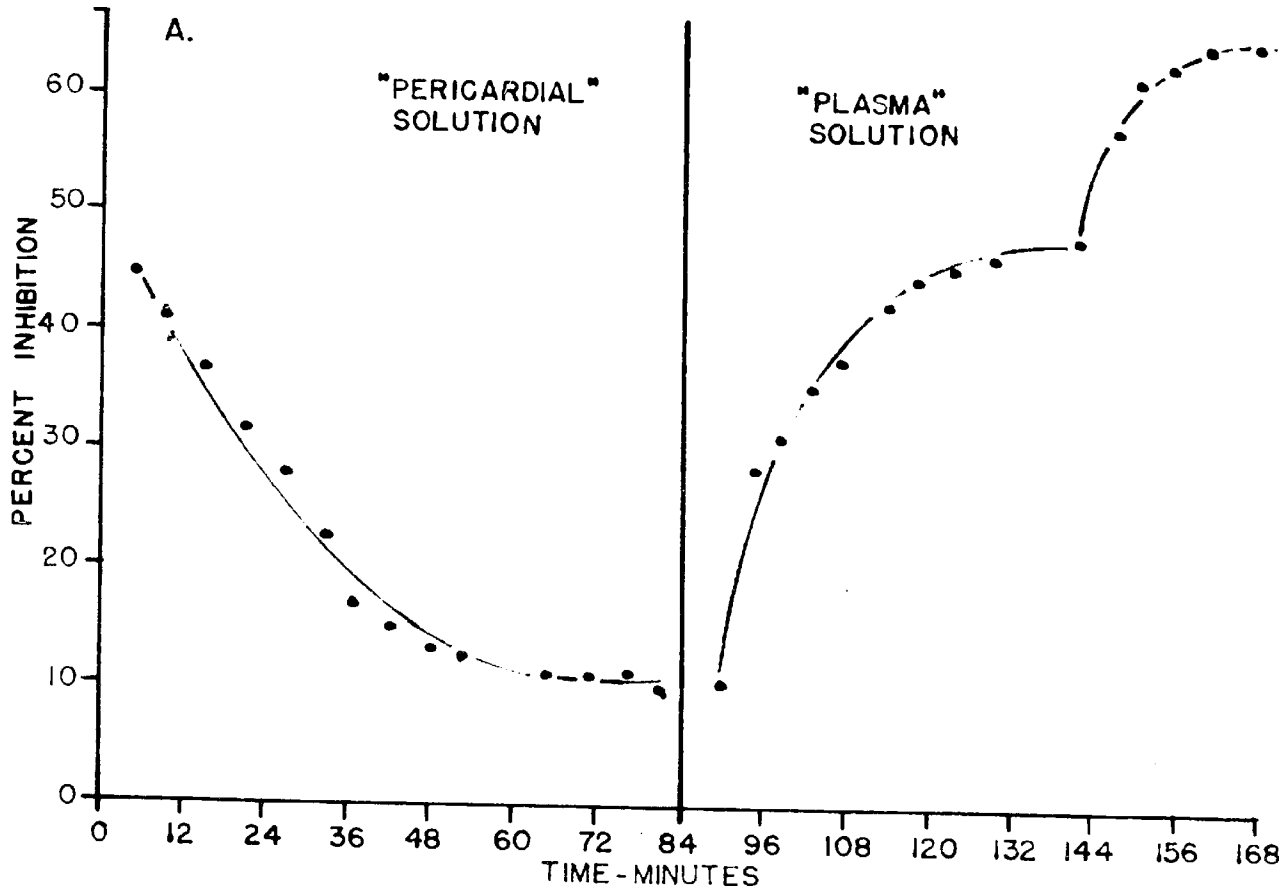

5 MINUTES
9
14
20.5
26
32
36.5 MINUTES
42.5
48
53.5
65
71
76 MINUTES
80.5
90
94
98
102
106.5 MINUTES
113.5
117.5
123.5
129
141
146 MINUTES
150
155.5
162.5
167.5

Figure 2. A. Percent of maximum inhibition for each vagal stimulation of figure 1 is plotted against the time of stimulation.
B. Natural auricular rate (determined just prior to vagal stimulation) plotted against time.


A.
"PERICARDIAL" SOLUTION
"PLASMA" SOLUTION
PERCENT INHIBITION
0
10
20
30
40
50
60
0 12 24 36 48 60 72 84 96 108 120 132 144 156 168
TIME - MINUTES

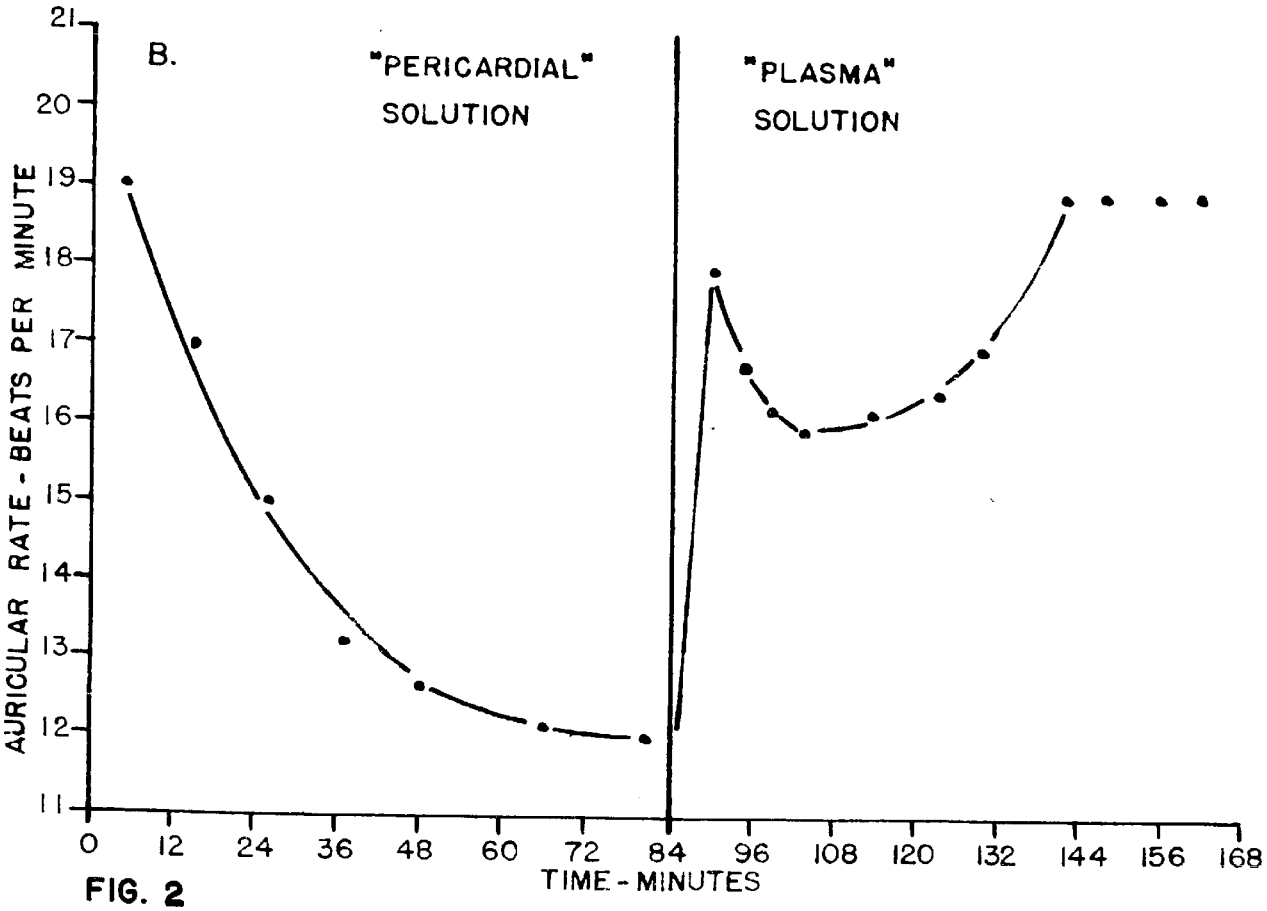

B.
"PERICARDIAL" SOLUTION
"PLASMA" SOLUTION
AURICULAR RATE - BEATS PER MINUTE
11
12
13
14
15
16
17
18
19
20
21
0 12 24 36 48 60 72 84 96 108 120 132 144 156 168
TIME - MINUTES

FIG. 2

Figure 3. Experiment showing the effect of cholinesterase on a single shock stimulation. Strength of stimulation was 40 volts. Duration of each stimulus was 0.2 milliseconds.
A. Control period obtained before cholinesterase was applied to the auricle.
B. Record starts 6 minutes after the application of cholinesterase. Cholinesterase was applied for 30 minutes.
C. Control following the removal of cholinesterase.

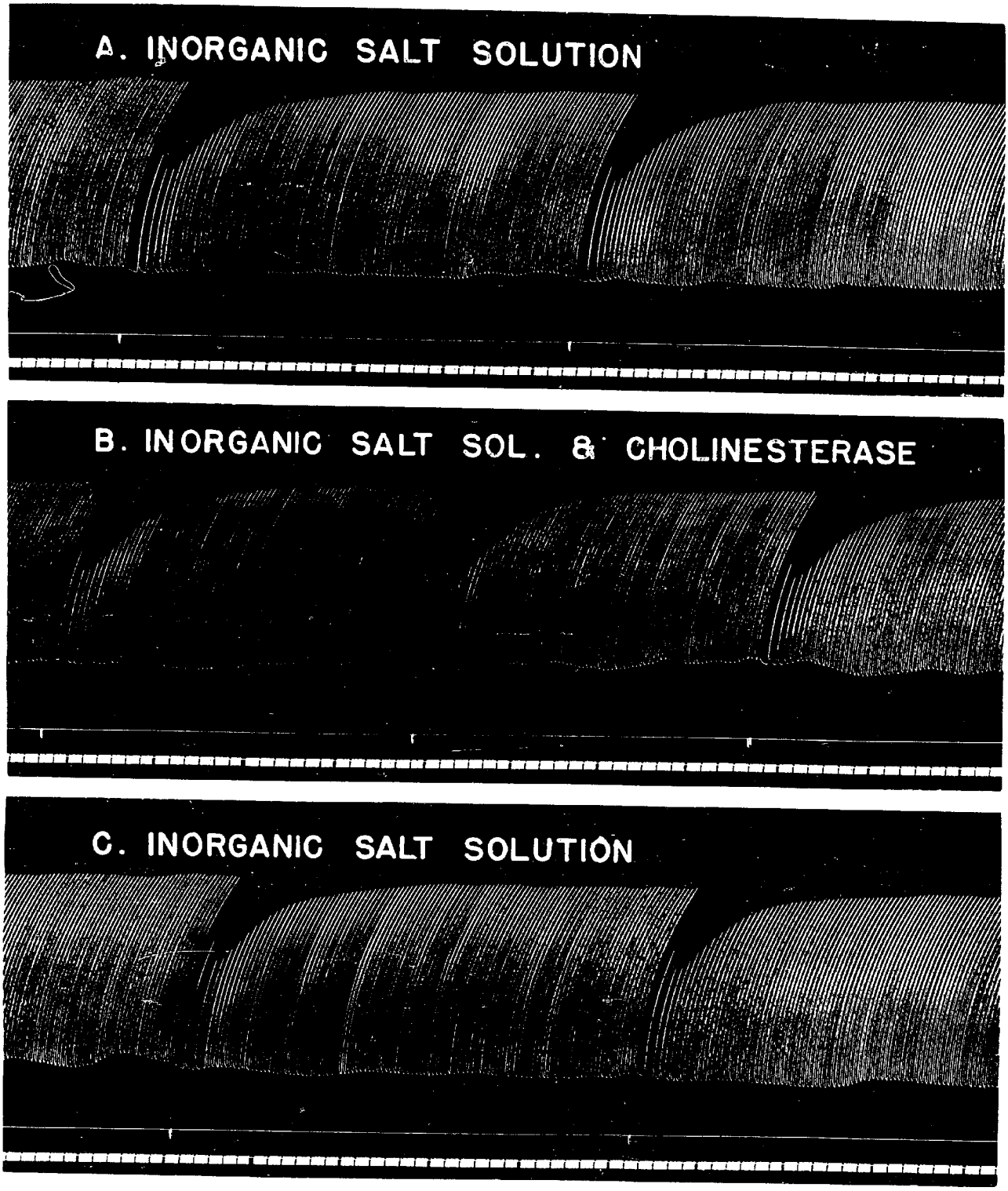

A. INORGANIC SALT SOLUTION
B. INORGANIC SALT SOL. & CHOLINESTERASE
C. INORGANIC SALT SOLUTION

FIG. 3

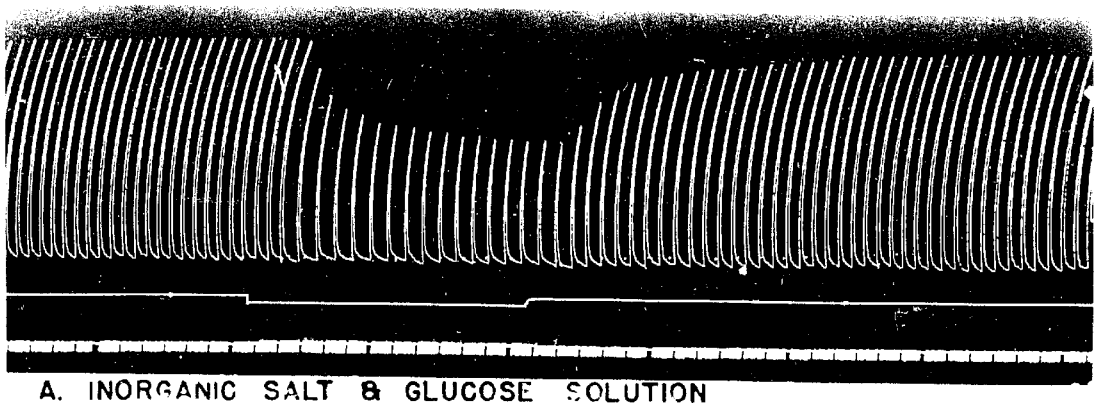
later heading.

If a multiple shock stimulation of submaximal strength is applied to the vagus nerve for a sufficient length of time, the inhibition of the auricle increases gradually until it reaches a maximum. When cholinesterase was applied to the rhythmically beating auricle, it was found that the maximal inhibition from the multiple stimulation of the vagus nerve was less than the control. This was a reversible phenomenon but it often took considerable time for complete reversal, and in many experiments, complete recovery was never achieved.

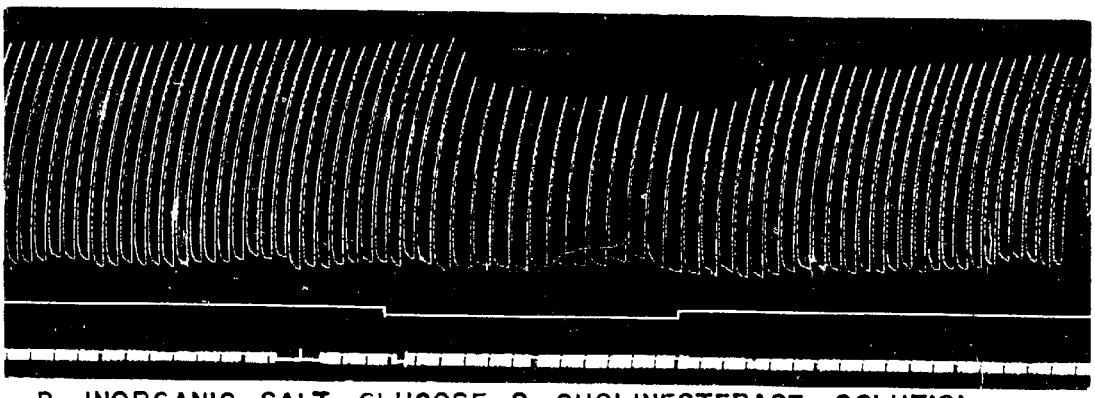
The effects of the cholinesterase fall into two classes, the inotropic effects and the chronotropic effects. The inotropic effect is manifest by less decrease in the height of contraction during vagal stimulation. The chronotropic effect is manifest in a smaller decrease in the rate of automatic beating during the stimulation as compared to the control stimulation before the cholinesterase. Inotropic effects always took less time to recover than chronotropic effects. Often time was not allowed for the chronotropic effect to recover if the object of the experiment was primarily to study inotropic effects. Chronotropic changes were never recorded without an inotropic change. However, if a very weak stimulus was used, an inotropic change could be produced without any signficant observable chronotropic change.

Inotropic effects due to cholinesterase may be observed in two representative experiments shown in figures 4 and 6. In these experiments the percentage of inhibition for any instant was computed by subtracting the height of the contraction at the instant desired (Hi) from the normal height of contraction before stimulation (Hn), dividing by the normal height of contraction and multiplying by 100.

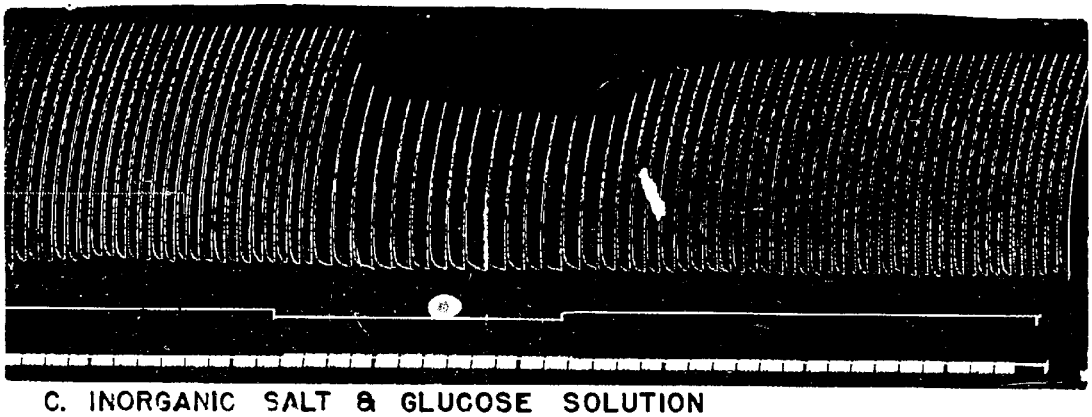
Figure 4. Experiment showing the inotropic effect of cholinesterase on multiple shock stimulation of the vagus nerve. Strength of stimulus was 40 volts. Duration of each single shock was 0.2 millisecond. Frequency of stimulation was 1 per 2 seconds.
A. Control period before cholinesterase.
B. Stimulation 21 minutes after the application of cholinesterase.
C. Control 6 minutes after the removal of cholinesterase.
D. Control 17 minutes after the removal of cholinesterase



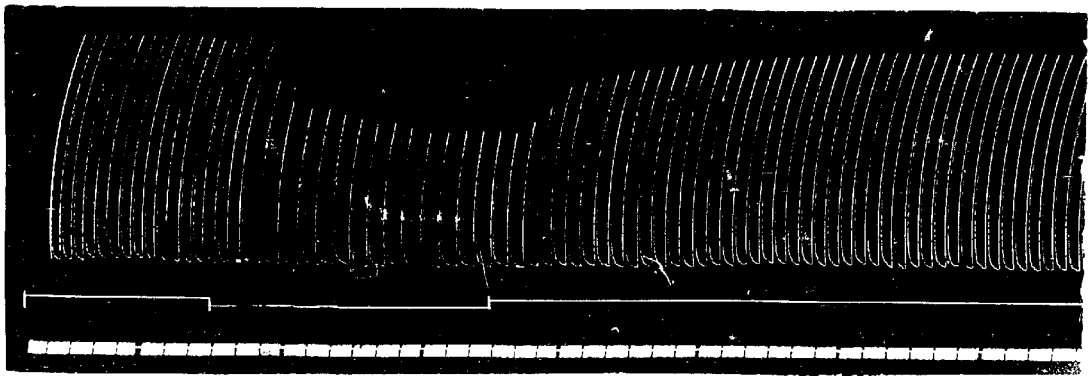
A. INORGANIC SALT & GLUCOSE SOLUTION



B. INORGANIC SALT, GLUCOSE & CHOLINESTERASE SOLUTION
21 MINUTES AFTER APPLICATION



C. INORGANIC SALT & GLUCOSE SOLUTION
6 MINUTES AFTER REMOVAL OF CHOLINESTERASE



D. INORGANIC SALT & GLUCOSE SOLUTION
17 MINUTES AFTER REMOVAL OF CHOLINESTERASE

FIG. 4

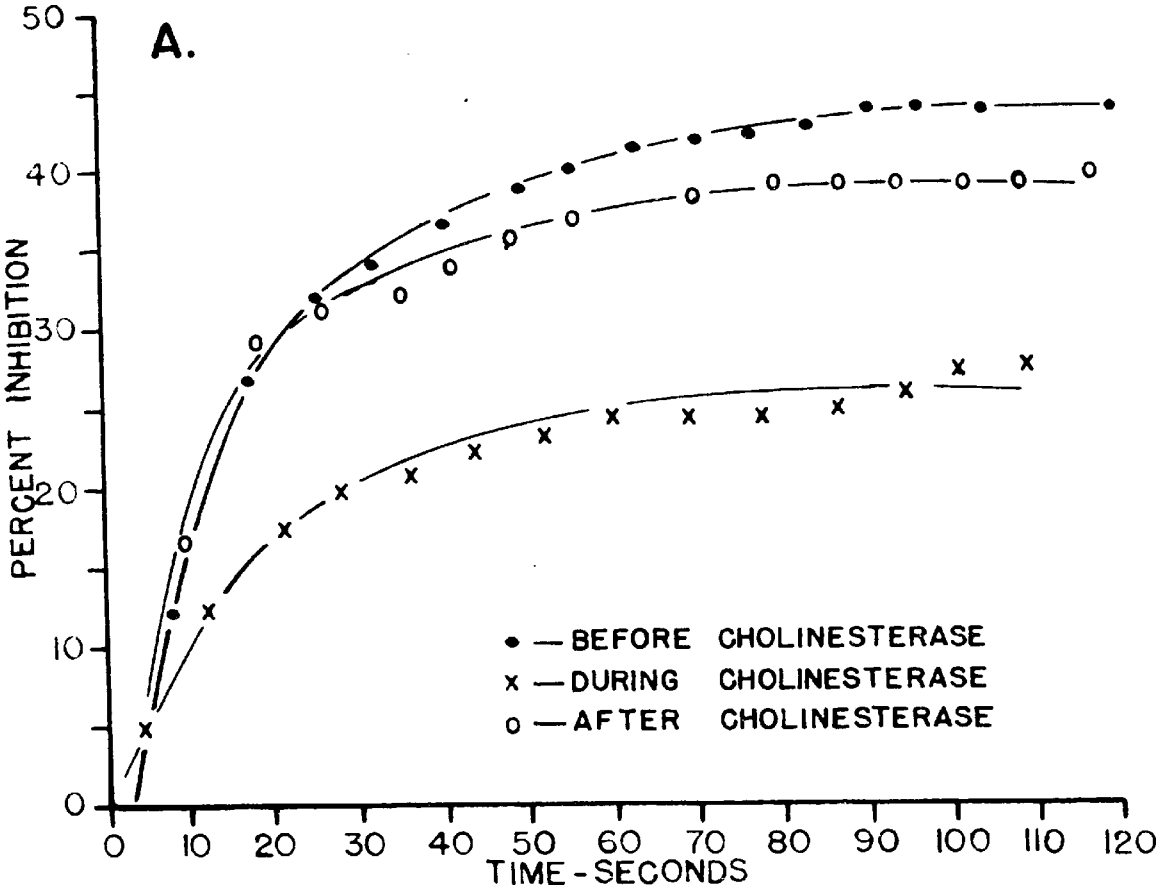
$$\frac{Hn - Hi}{Hn} \times 100 = \text{percent inhibition}$$

Figure 4 shows the inotropic effect of the cholinesterase when the stimulus used was 40 volts lasting 0.2 millisecond and applied 1 shock per 2 seconds for a period of 120 seconds (60 shocks). The percent inhibition of each beat of the auricle following stimulation was plotted against time (figure 5). This was done for the control period before cholinesterase, for the period 21 minutes after the cholinesterase was applied, and a recovery control period 17 minutes after the cholinesterase was removed.

It is seen (figure 5A) that the percentage inhibition of the control before the cholinesterase increased until it reached a constant level of about 44 percent. Twenty-one minutes after the application of the cholinesterase, vagal stimulation with the same stimulus produced the maximal inhibition of about 27 percent. This is a 39 percent decrease in the inhibition. Seventeen minutes following the removal of the cholinesterase and replacing with fresh inorganic salt solution, vagal stimulation caused 40 percent inhibition. Thus, the auricle failed to show complete recovery during the time of recording. Inhibition curves were not plotted for figure 4C, but the record was included to show the progressive phase of recovery.

Figure 6 shows the results of another experiment also conducted to study the inotropic effects of cholinesterase. Three different strengths of stimulation were used, 10, 20, and 40 volts (figure 6A, B,and C, respectively). These were multiple shock stimuli, each of the single shocks lasting 0.2 millisecond. They were applied to the vagus at a rate of 1 shock per 2 seconds for a period of 120 seconds (60 shocks). The percent inhibition of certain beats of the auricle during the stimulation was plotted against time for

Figure 5. A. Percent of inotropic inhibition plotted against time for each of the beats that occurred during the vagal stimulation.
B. Percent of inotropic inhibition plotted against time for each of the beats that occurred during the recovery after vagal stimulation.


A.
PERCENT INHIBITION
TIME - SECONDS
• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE

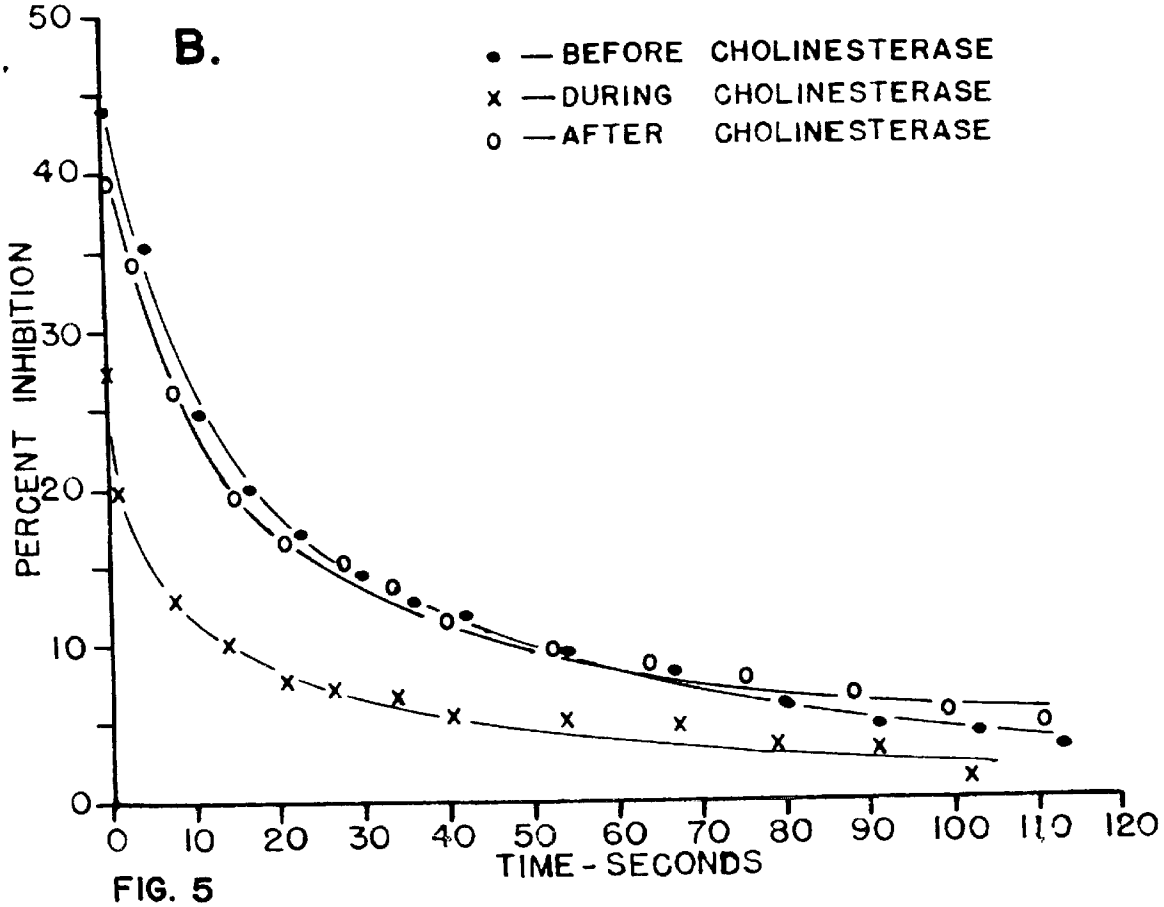
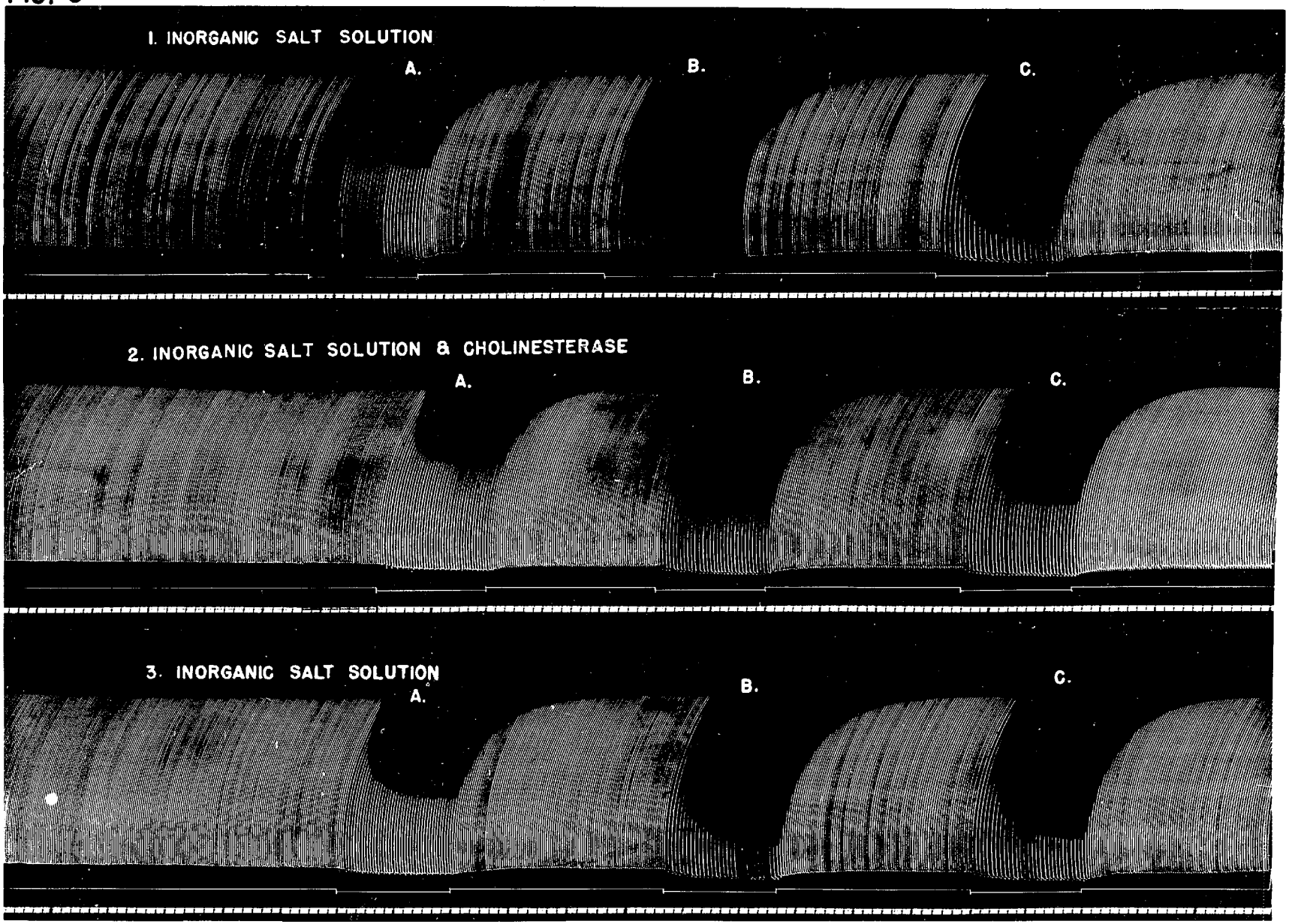

B.
PERCENT INHIBITION
TIME - SECONDS
• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE

FIG. 5

Figure 6. Experiment showing the inotropic effect of cholinesterase on different strengths of stimulation.
A. Strength of stimulation was 10 volts.
B. Strength of stimulation was 20 volts.
C. Strength of stimulation was 40 volts.
The duration of each of the single shocks was 0.2 millisecond. The frequency of stimulation was 1 per 2 seconds.

FIG. 6


1. INORGANIC SALT SOLUTION
A.
B.
C.
2. INORGANIC SALT SOLUTION & CHOLINESTERASE
A.
B.
C.
3. INORGANIC SALT SOLUTION
A.
B.
C.

each strength of stimulation for the control period before cholinesterase, for the period during cholinesterase, and for the recovery period after cholinesterase (figures 7A, 8A, and 9A). Table 1 shows the percent of maximum inhibition produced with each of the three strengths of stimulation for the three periods before, during, and after cholinesterase.

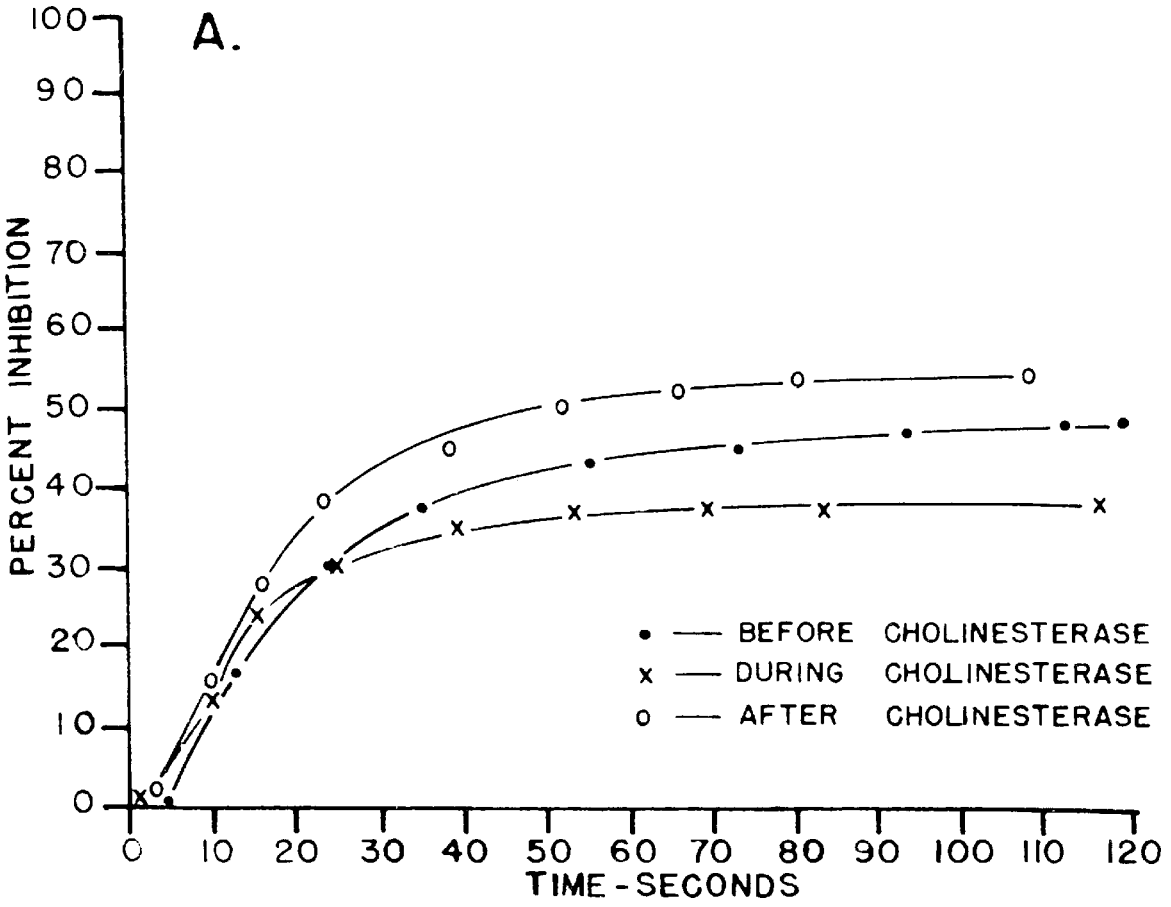
TABLE 1

Strength of stimulus	Percent of maximum inhibition		
	Before cholinesterase	During cholinesterase	After cholinesterase
10	48.6	38.2	54.9
20	89.5	64.3	79.2
40	86.9	59.6	76.0

It is seen that the 40 volt stimulation produced less inhibition than the 20 volt stimulation. The electrodes were reversed to determine if this was due to an electrotonus resulting from the direction of current flow. No change was noted (record not shown). It was then inferred that the stronger stimuli might be stimulating accelerator fibers, thus cancelling part of the action of the cholinergic fibers (Pannier, 1946).

In certain cases, illustrated in figure 10, an auricular preparation was obtained which was more sensitive to chronotropic changes. The strength of the stimulus in this experiment was 10 volts, lasting 0.2 milliseconds, and was applied 1 shock per 2 seconds for 60 seconds (30 shocks). Before the cholinesterase was applied this stimulation resulted in complete inhibition, which started immediately after the stimulus was applied and ended only when the stimulation was stopped. After the cholinesterase was administered, the chronotropic effect was readily observed as the stimulus no longer caused complete inhibition but allowed some beats to escape. Four

Figure 7. The percent of inotropic inhibition plotted against time for the experiments shown in figure 6A.
A. For the period of the vagal stimulation.
B. For the period of recovery following the vagal stimulation.


A.
PERCENT INHIBITION
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100 110 120
TIME - SECONDS
• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE

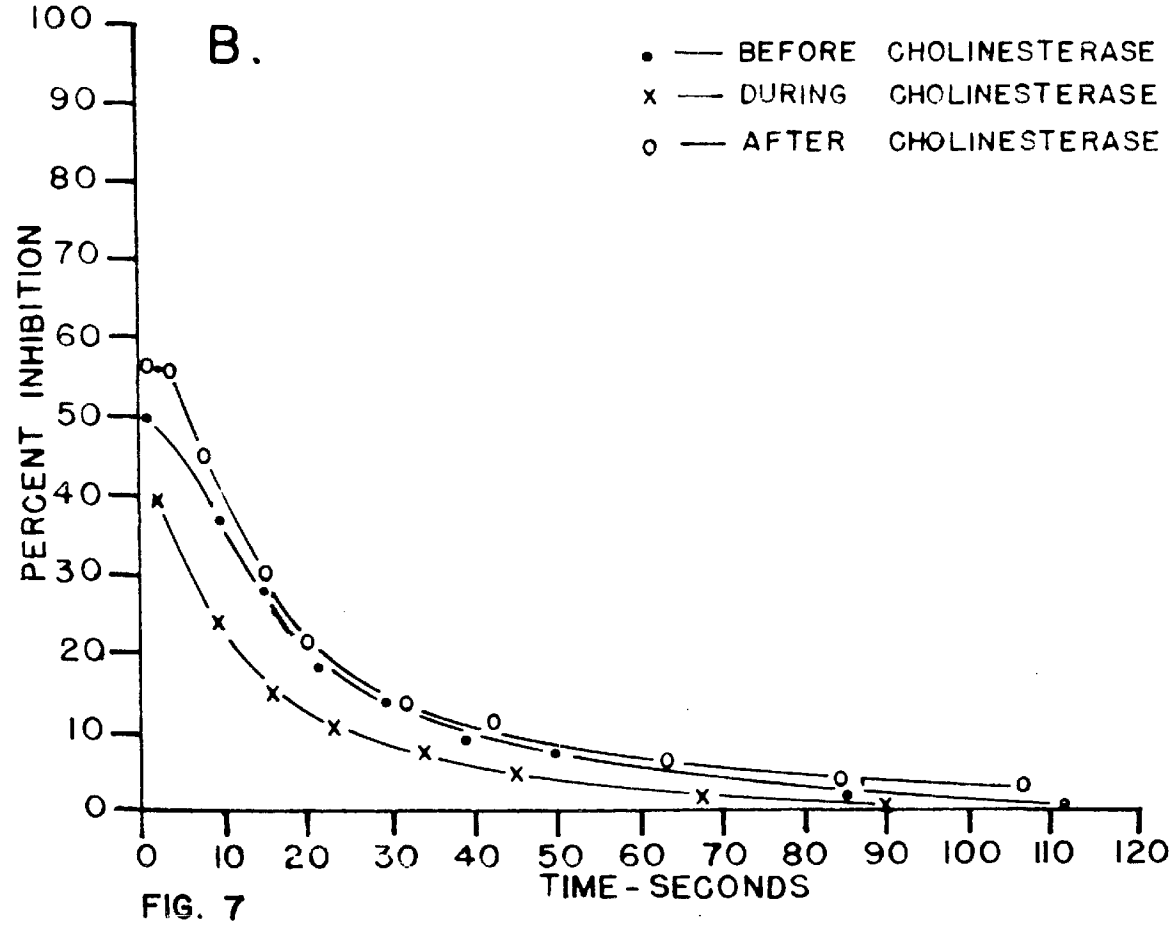
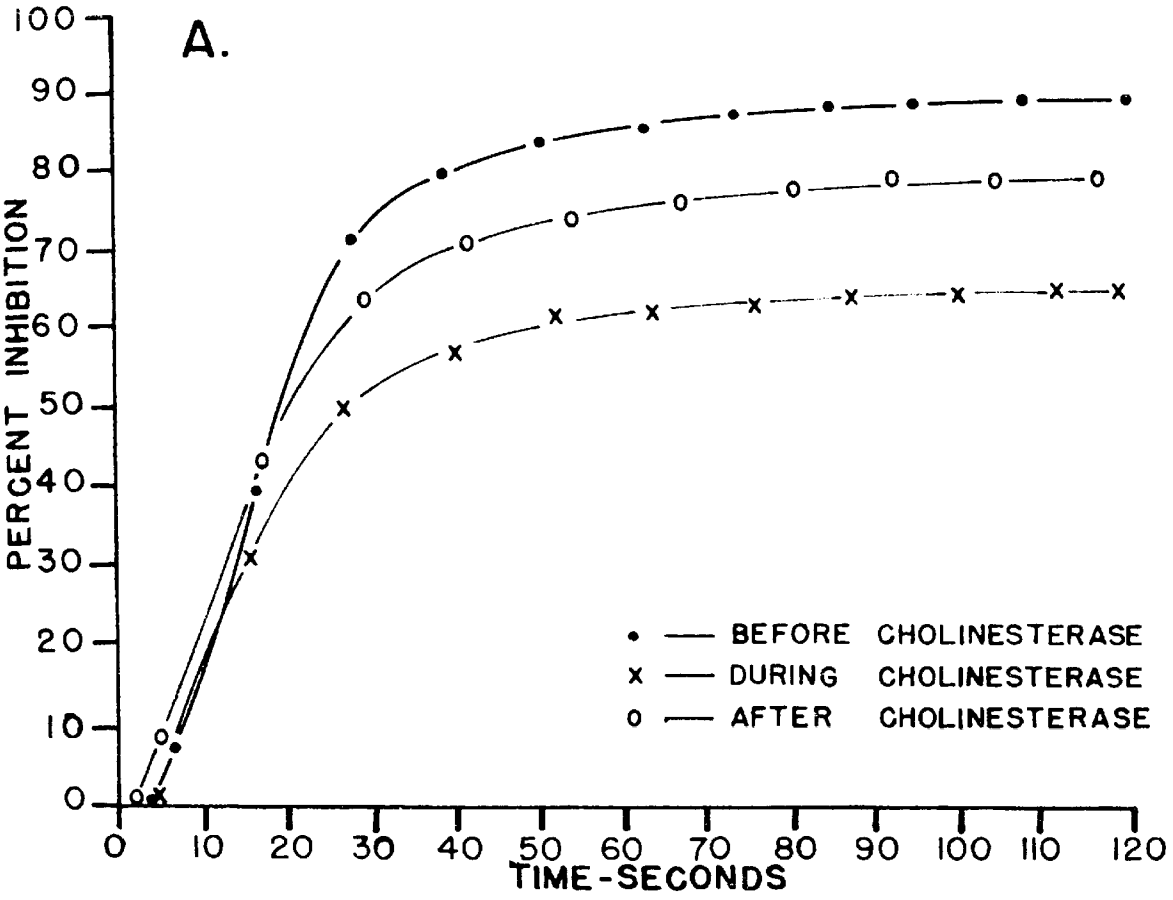

B.
PERCENT INHIBITION
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100 110 120
TIME - SECONDS
• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE

FIG. 7

Figure 8. The percent of inotropic inhibition plotted against time for the experimental procedure shown in figure 6B.
A. For the period of vagal stimulation.
B. For the period of recovery following the vagal stimulation.


A.
PERCENT INHIBITION
TIME-SECONDS
• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE

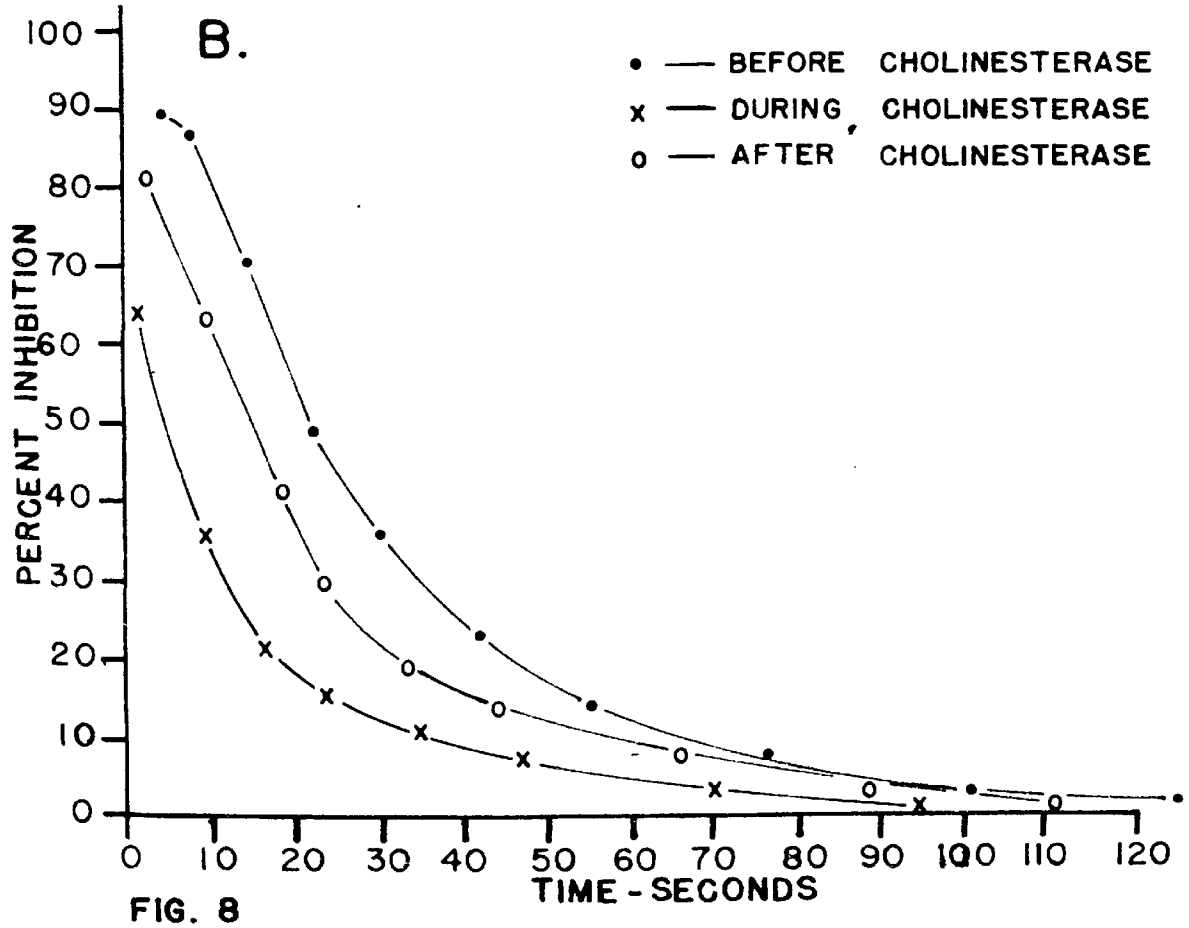
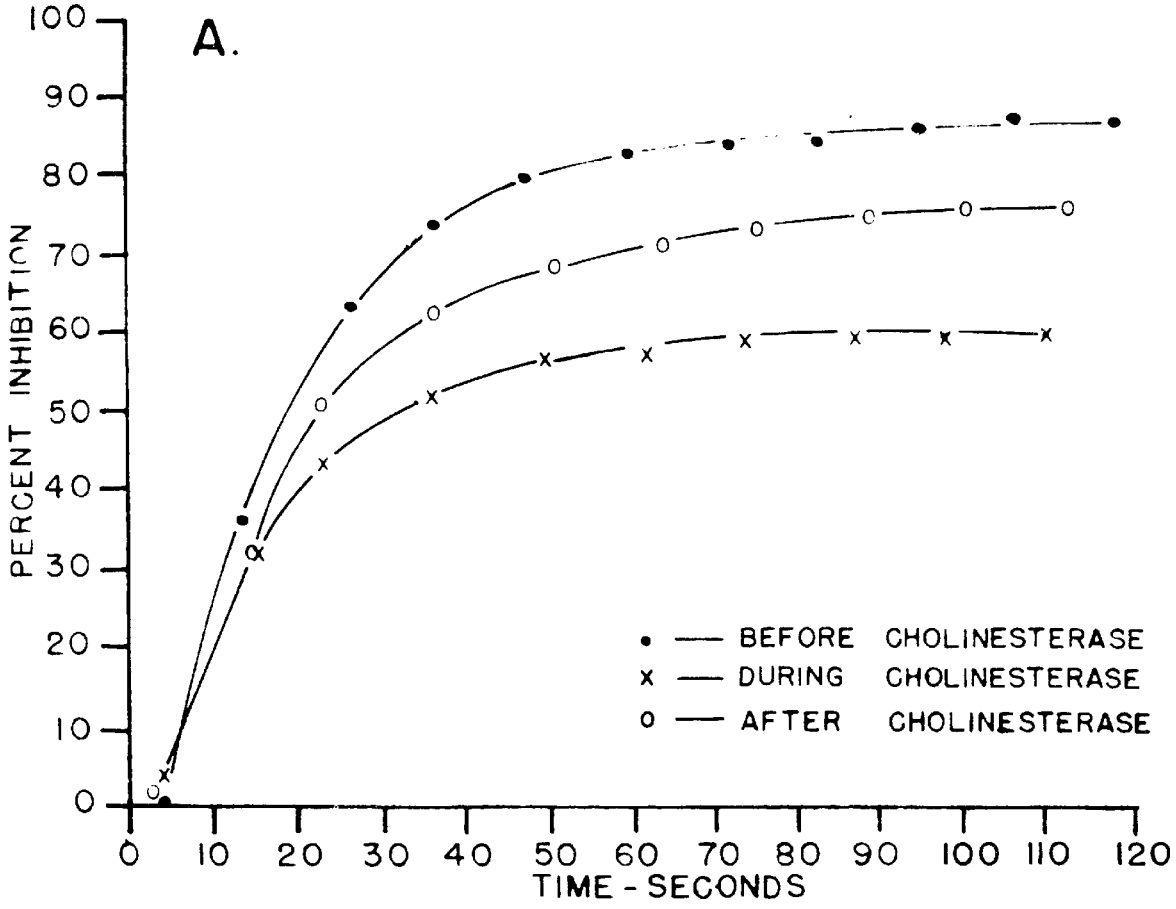

B.
PERCENT INHIBITION
TIME-SECONDS
• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE

FIG. 8

Figure 9. The percent of inotropic inhibition plotted against time for the experimental procedure shown in figure 6C.
A. For the period of vagal stimulation.
B. For the period of recovery following the vagal stimulation.


A.
PERCENT INHIBITION
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100 110 120
TIME - SECONDS
• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE

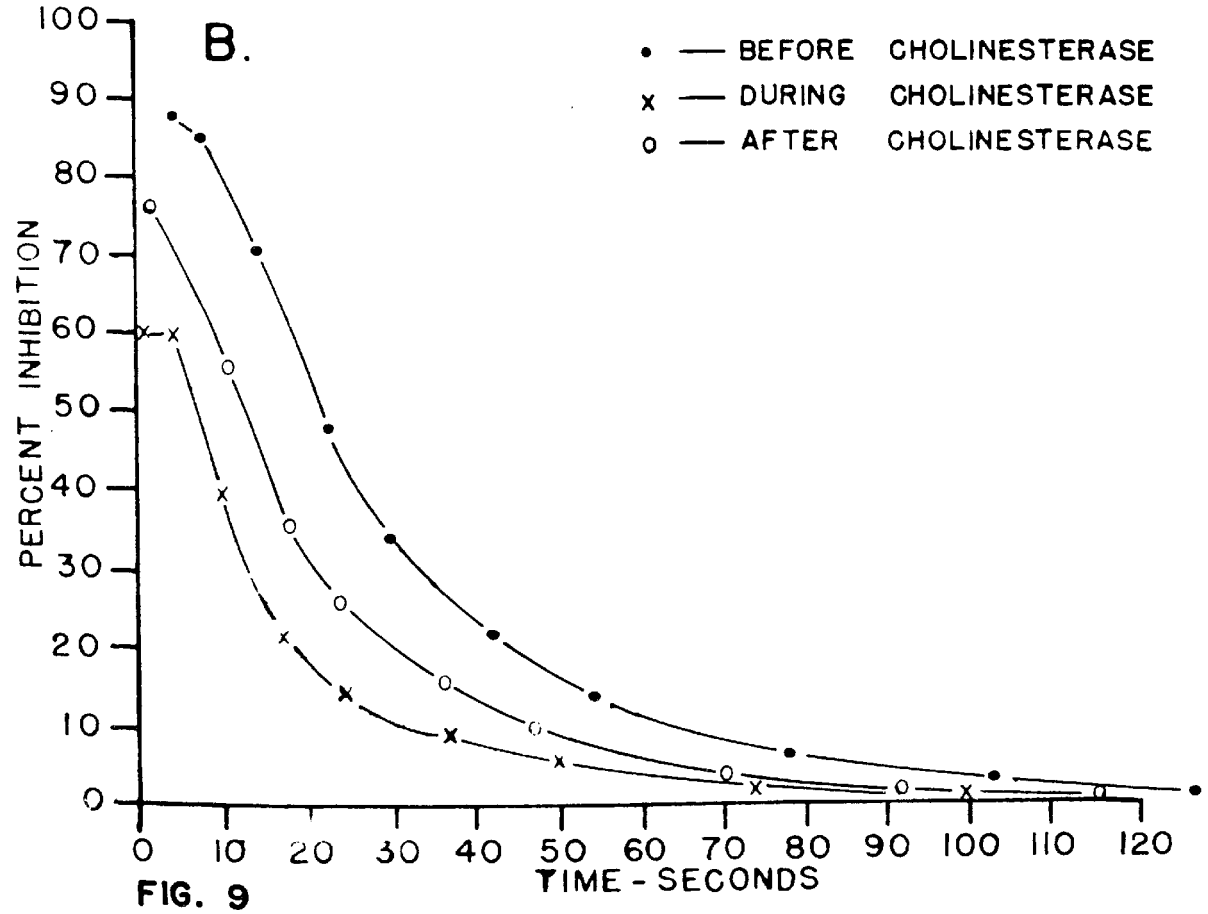
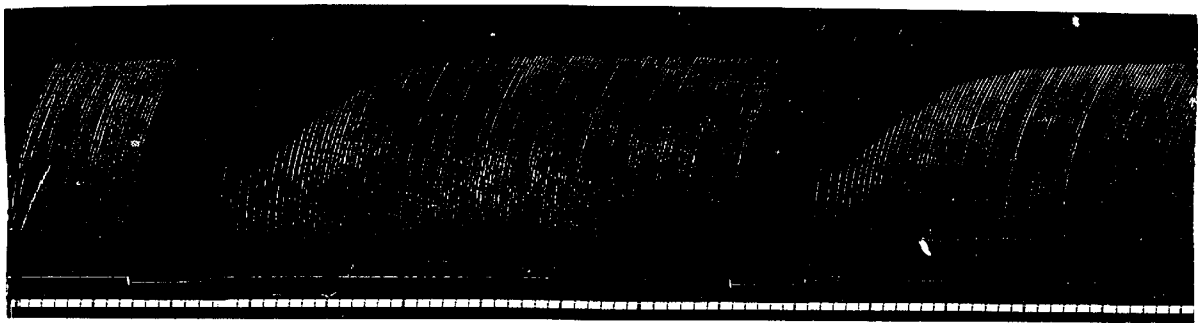
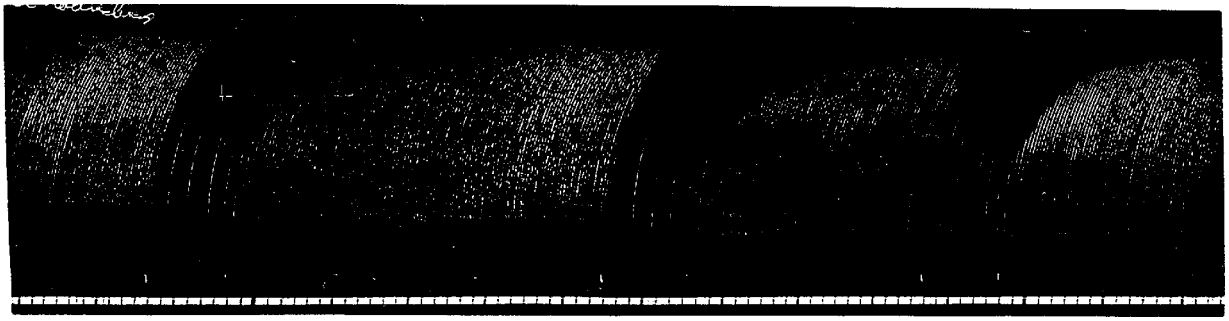

B.
PERCENT INHIBITION
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100 110 120
TIME - SECONDS
• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE

FIG. 9

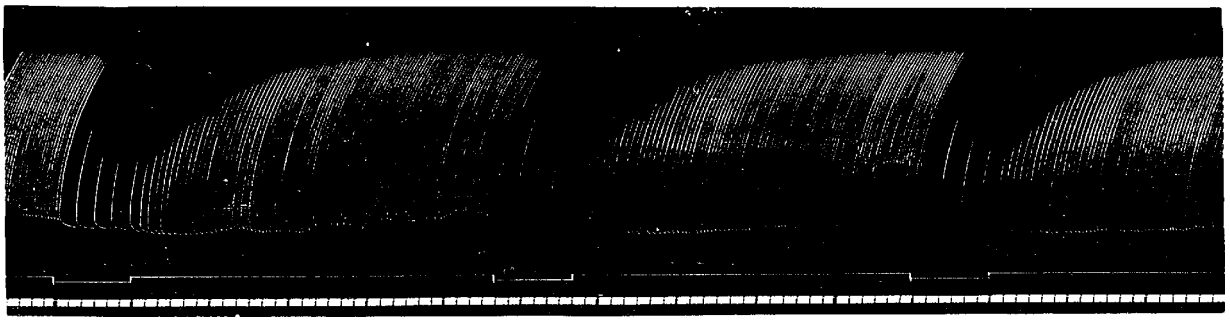
Figure 10. Experiment showing the chronotropic effect of cholinesterase when the vagus nerve is stimulated with a multiple stimulus. Strength of each shock was 10 volts. Duration of each shock was 0.2 milliseconds. Frequency of stimulation was 1 per 2 seconds.



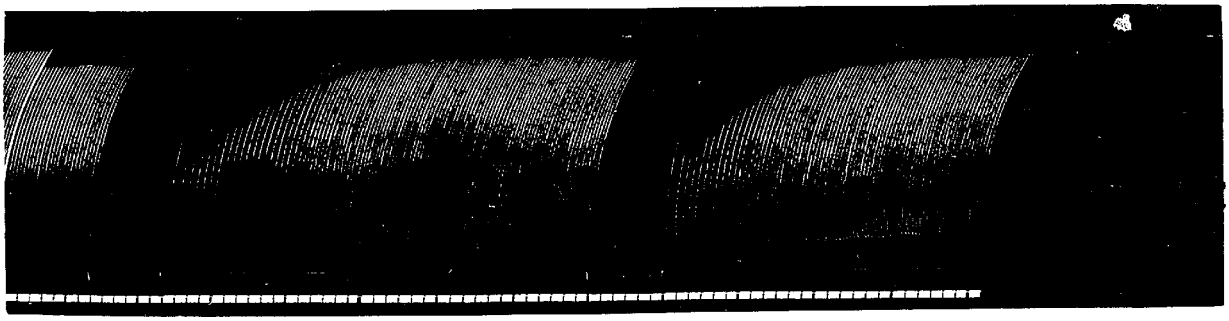
A. INORGANIC SALT SOLUTION



B. INORGANIC SALT SOLUTION & CHOLINESTERASE APPLIED FOR 18 MINUTES.



C. INORGANIC SALT SOLUTION
8-24 MINUTES AFTER CHOLINESTERASE REMOVAL



D. INORGANIC SALT SOLUTION
45-57 MINUTES AFTER CHOLINESTERASE REMOVAL

FIG. 10

minutes after the cholinesterase was first applied, the vagus was stimulated, and the auricle beat 4 times during the stimulation. Ten minutes after the application of cholinesterase vagal stimulation allowed 5 beats to occur; fifteen minutes after application vagal stimulation allowed 6 beats to occur. Because of the long time required for recovery, the cholinesterase was removed after this brief demonstration of chronotropic effect. Nine minutes after removing the cholinesterase by replacement with fresh solution, the auricle began to show recovery (figure 10C). Vagal stimulation allowed 5 beats to escape. Fifteen minutes later, only four beats escaped. Twenty minutes decreased it to three. With another change to fresh solution, the vagal stimulation again caused complete inhibition (figure 10D). This was 45 minutes after the removal of the cholinesterase.

Whenever a stimulus was stopped, whether it was a single shock or a multiple shock, the inhibition of the auricle gradually decreased until the height of the contraction was the same as before the stimulus was applied (any record). The percent inhibition for figures 4 and 6 were plotted against time to give the curves showing the recovery of the auricle from the stimulus (figures 5B, 7B, 8B, and 9B).

Discussion

When the right vagus nerve of an isolated turtle auricle is stimulated, inhibition of the contraction of the auricle occurs. As recorded on the kymographion by a muscle lever attached to the auricle, both the amplitude of the beat and the rate of the beating decreased. With strong stimulation, the contraction of the auricle will completely stop for a period of time. This inhibition has been shown to be due to the action of acetylcholine or a similar substance

released in the sinus venosus and in the auricle (Loewi, 1921, 1922; Clark, et al, 1938; Lissak, et al, 1942). Also, as was shown by Dale (1914 a) and innumerable times since, acetylcholine applied directly to the heart causes inhibition of its action.

After the acetylcholine is released the heart must eliminate the substance if it is to return to normal. It has been reasoned by Brown, et al, (1936) that the acetylcholine of skeletal muscle must be hydrolyzed within the refractory period of the muscle if a second separate of the muscle is to occur. This does not apply to the inhibitive property of the vagus nerve on the heart, since it is obvious from the records that the inhibition is not dissipated within the refractory period of one beat but extends over several such periods. Therefore, cholinesterase does not have to be present in such large quantities. The heart tissue is assumed to be a homogeneous solution of cholinesterase activity. However, the enzyme is estimated to have a very large molecular weight and, due to the characteristics of such substances, it may be concentrated at the surface of the cell. This does not change in any way the following considerations.

The degree of inhibition of the auricle can be altered by merely varying the strength or frequency of the stimulus applied to the vagus nerve. Single shocks of different strength may cause inhibition varying from the threshhold amount to complete inhibition. If a single shock of a strength sufficient to cause a just visible amount of inhibition to occur is applied frequently enough (multiple stimulation), the inhibition increases until a constant level is reached (figures 4 and 6). Considering this in terms of the acetylcholine-cholinesterase system, a small amount of acetylcholine is liberated in the auricle when the vagus nerve is stimulated with a single shock. If several such shocks (multiple stimulation) are

applied, each shock liberates a quantity of acetylcholine in the auricle.

The amount of acetylcholine liberated in the auricle with each single shock may be considered as being released at the same time. If the conduction time for the vagus nerve is taken as 50 centimeters per second (Heinbecker, 1930) and the difference between the shortest and the longest vagal fibers to the auricle is an extreme estimate of 2 centimeters, then the difference in the time of the release of the acetylcholine between the longest and shortest fibers can only be approximately 0.04 second. This is an insignificant time as compared to the 10-25 seconds (Fredericq, 1938; This author) that it takes for the maximum inhibition to occur with a single shock. Thus, it is unlikely that the curve showing the gradual increase to maximum inhibition with the single shock is due to different lengths of the vagus nerve.

Each of the quantities of acetylcholine is assumed to be liberated within the same area as the previous one. This is a logical assumption since the strength of stimulation is constant and the area of the nerve in contact with the electrode is constant. The time between the stimuli (2 seconds) is sufficiently long that one stimulus will not interfere with another since the refractory period is probably, at its maximum, 0.5-5.0 milliseconds (Fulton, 1950; Prosser, 1950; Fredericq, 1938; Brown, 1934).

Two main factors would contribute to the curves showing the increase to maximum inhibition. One of these is the hydrolysis of the acetylcholine and the other is the beating of the auricle to distribute the acetylcholine.

Hydrolysis of the acetylcholine starts immediately after the release of the first quantity of acetylcholine from a vagal stimulation. Before the second quantity is released by a second stimu-

lation, a certain fraction of the first quantity will have been destroyed. Since both quantities were liberated in essentially the same area, the second quantity will add to the remainder of the first. Successive stimuli add increments to the existing level of acetylcholine until a point is reached when the amount of acetylcholine released with each stimulus is the same as the amount hydrolyzed in the period of time between the two stimuli. When these two quantities are equal, the level of the acetylcholine will remain constant in the auricle.

It is possible that the inhibitive action of the acetylcholine might follow Langmuir's theory of heterogeneous reactions (Glasstone, 1946). One could consider the contractile unit as a surface with prosthetic groups. Acetylcholine could produce inhibition by reacting with the prosthetic group to interfere with the contractile process. That is, the inhibition would depend on the deactivation of some unit of the contractile process. Deactivation would then be a function of the concentration of acetylcholine and the area available to the acetylcholine.

The concentration of acetylcholine depends on the rate of hydrolysis. If the rate of hydrolysis is increased by the addition of cholinesterase, the concentration of the acetylcholine cannot increase to as high a level before the amount hydrolyzed is equal to the amount released by each single shock. Thus, the amount of inhibition will decrease as the rate of hydrolysis increases. If one assumes a constant rate of hydrolysis, the curves (figures 5A, 7A, 8A, and 9A) obtained showing the gradual increase of inhibition to a maximum may be explained as did Clark and Raventos (1938). At any specific time, T_1, a quantity of acetylcholine, X, is liberated. After a constant interval and at time, T_2, the quantity, X, is also liberated. The second quantity will add to the amount remaining of

the hydrolyzing first quantity. This will continue at each time, T, until the amount being hydrolyzed in the constant interval between the times, T, is equal to the amount released with each stimulus. Then the maximum inhibition will occur.

If the rate of hydrolysis is taken as 0.1 of the amount of acetylcholine present, the increase of acetylcholine level can be shown mathematically as follows:

TABLE 2

T = Time with equal intervals
X = Amount of acetylcholine liberated with each stimulus.

T	X	T	X
T_1	X	T_{20}	8.8X
T_2	X + (X - 0.1X) = 1.9X	T_{25}	9.3X
T_3	X + (1.9X - 0.2X) = 2.7X	T_{30}	9.6X
T_4	X + (2.7X - 0.3X) = 3.4X	T_{35}	9.8X
T_5	X + (3.4X - 0.3X) = 4.1X	T_{40}	9.9X
T_{10}	6.5 X	T_{45}	9.9X
T_{15}	7.9X	T_{50}	10.0X

The plotted points of figure 11 are taken from the experiment shown in figure 6B. The curves drawn through these points are determined by the theoretical method based on the idea presented above. The curve before the cholinesterase was based on 10 percent hydrolysis in the time interval between stimuli. The curve after cholinesterase was based on 12.5 percent hydrolysis in the same time interval.

Adding cholinesterase to the preparation resulted in a decrease of inotropic inhibition. The rate of hydrolysis was increased, destroying a larger fraction of the acetylcholine in the same interval between successive stimuli than before the cholinesterase. This means that there was a decrease in the time required for the

Figure 11. The points plotted, taken from the data shown in figure 6B, show the gradual increase of inhibition with multiple shock stimulation. The line drawn through the points is the computed line based on the accumulation of acetylcholine in the auricle.

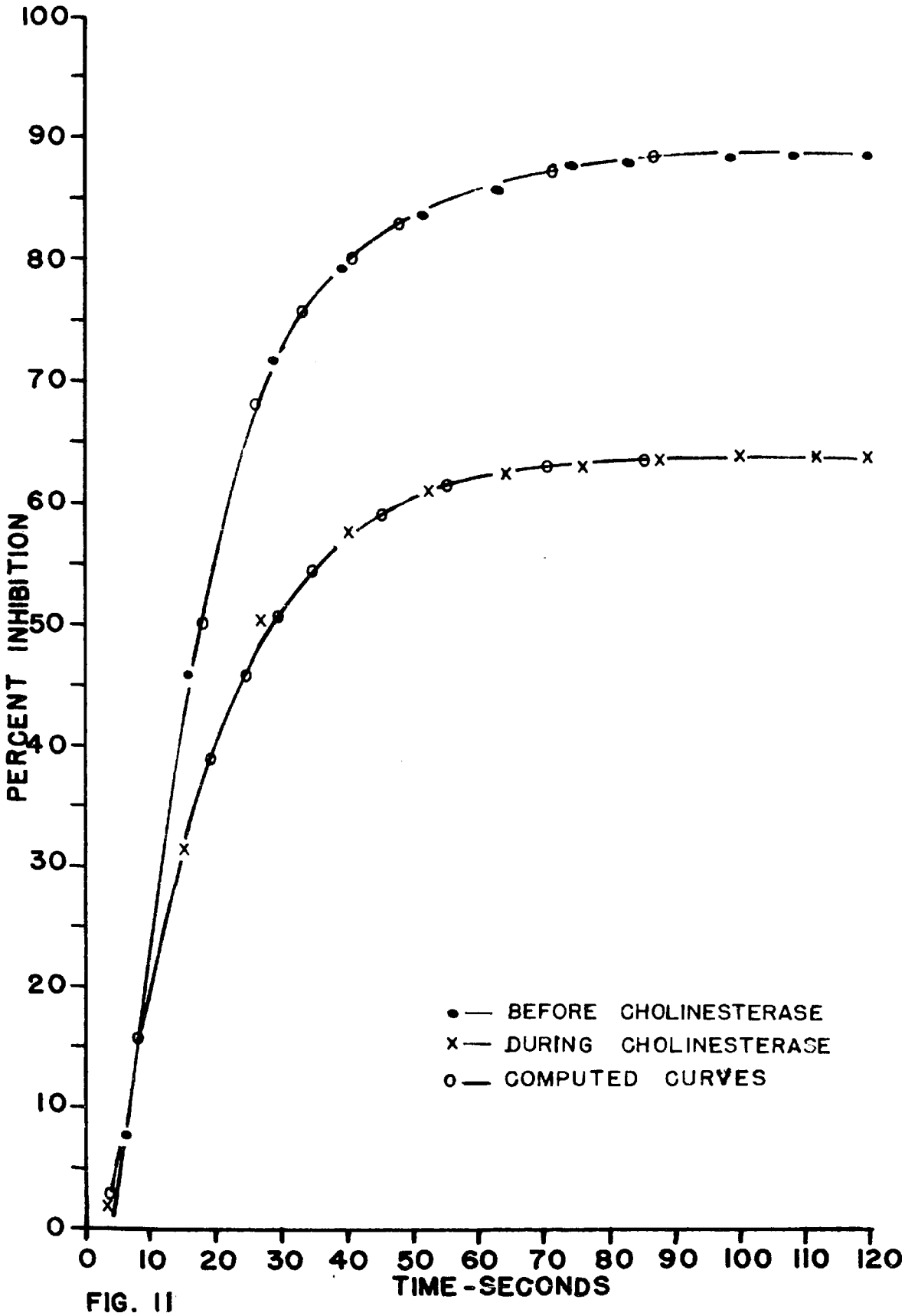

100
90
80
70
60
50
40
30
20
10
0
PERCENT INHIBITION
0 10 20 30 40 50 60 70 80 90 100 110 120
TIME-SECONDS
● — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — COMPUTED CURVES

FIG. 11

amount of acetylcholine released with each stimulus to equal the amount hydrolyzed in the time interval between successive stimuli. In the representative experiment shown in figure 6B, the time required for the maximum inhibition to occur before the cholinesterase was 89 seconds. After the cholinesterase, it was 64 seconds. Since there was a decrease in time for maximum inhibition to occur, there was a decrease in the number of stimuli that could be applied before the equilibrium point. Thus, the concentration of acetylcholine was not as great after equilibrium was established and this resulted in a decreased inhibition of the auricle.

If the amount of inhibition depends on the deactivation of the prosthetic group or some similar process, the area available to the acetylcholine will be a factor. The surface area involved in a vagal stimulation depends on the strength of stimulus and on the diffusion of the acetylcholine, both simple diffusion due to concentration gradients and diffusion due to the force applied by the beating of the auricle.

It is commonly known that increasing the strength of the stimulus to the vagus increases the amount of inhibition from threshhold to a maximal amount. This increased strength increases the number of nerve fibers stimulated. Thus, acetylcholine will be liberated by a larger number of fibers over a larger area with the greater stimulation.

The diffusion of acetylcholine obviously increases the area available for reaction. Diffusion due to concentration gradients is a difficult quantity to determine in this type of system. Although this diffusion is surely present, the active forcing of acetylcholine over a larger area by the beating of the auricle must add a greater effective force. The following paragraphs will demonstrate the effect of the beat of the auricle on the amount of inhibition

produced with vagal stimulation.

The inhibition that occurs on a single shock stimulation to the vagus does not reach a maximum value immediately. It increases gradually for several beats until a maximum inhibition is attained in about 10-25 seconds. It has been suggested that the amount of acetylcholine is increasing to a maximum during the period that the auricle is being inhibited. For this explanation to be valid, the nerve, presumably the postganglionic fiber, would have to be continually firing for the period of 10-25 seconds after a single shock stimulation, 0.2 millisecond duration, had been applied. Heinbecker (1930) has shown that a single shock stimulation to the preganglionic vagal nerve produced only a single impulse down that nerve to the postganglionic cell. That a single shock of 0.2 millisecond duration could cause an after-discharge is doubtful. Larrabee and Bronk (1938) found in the sympathetic ganglion that a stimulus applied to the preganglionic fiber for a brief period of time at a frequency below 20 per second caused only a single response in the postganglionic fiber. The highest frequency used in these experiments was 1 shock per 2 seconds. Also, if the delay of maximum inhibition were due to the increase (after-discharge) of acetylcholine deposited in the auricle following the single shock stimulation, an increase of the cholinesterase concentration should increase the rate of destruction of acetylcholine. This would give less inhibition with the stimulation of the vagus nerve. Figure 3 shows single shock stimulation before, during, and after cholinesterase had been applied to the auricle. The amount of inhibition increased with the cholinesterase rather than being decreased as would be required by the after-discharge theory. Continual firing of the postganglionic fibers of the vagus to the auricle for the period of 10-25 seconds seems unlikely with a stimulus of such short

duration.

Since the logic of previous information indicates that after-discharge will not account for the delay of maximum inhibition, subsequent experiments on the turtle auricle were examined to provide a satisfactory explanation. These experiments produced evidence that the delay of maximum inhibition with a single shock stimulus was due to physical forcing of the acetylcholine by the automatic beating of the auricle into parts of the auricle that had not already been acted upon. This serves to disperse the liberated acetylcholine over a larger area and thereby produced more inhibition.

Figure 1 shows the results of single shock stimulation of the vagus when first "pericardial" solution is applied and then "plasma" solution. "Pericardial" solution has about 2.5 times as much sodium bicarbonate as the "plasma" solution (Table 4, appendix). The pH of the "pericardial" solution is 8.15 and that of the "plasma" solution is 7.75 as measured on a Coleman pH meter. The effect of vagal stimulation on the auricle with the more alkaline "pericardial" solution was to decrease the amount of maximum inhibition from 47 percent to 10 percent. When the relatively acid "plasma" solution was placed on the auricle, vagal stimulation (single shock) caused progressively more inhibition, starting at 10 percent and increasing until it caused 65 percent. One may infer that an increase in bicarbonate has increased the destruction rate of acetylcholine, thus causing less inhibition. This is consistent with the material presented by Gesell, Mason, and Brassfield (1944), who found that increasing the carbon dioxide increased the inhibition of the turtle auricle.

It was also noted that when the "pericardial" solution is placed on the auricle, the natural frequency of the auricular beat

decreased from 19 beats per minute to 12 beats per minute. Subsequent placing of "plasma" solution on the auricle caused the automatic beat to increase from 12 beats per minute to 19 beats per minute. This may be the same phenomenon that Bulbring and Burn (1949) observed. They allowed an excised cat's auricle to beat until the automatic rhythm stopped. Then, by applying a minute concentration of acetylcholine, the auricle would again beat. They decided that the auricle required a minimal amount of acetylcholine to maintain its automatic activity. When the alkaline "pericardial" solution was placed on the turtle auricle, the acetylcholine was more readily destroyed and the rhythm slowed as the amount of acetylcholine necessarily decreased. When the relatively acid "plasma" solution was placed on the auricle, the acetylcholine reached a more optimal level for the automatic beating. This suggests that the amount of acetylcholine in the auricle affects the automatic rhymicity. More work would have to be done to confirm any connection between the work of Bulbring and Burn and these experiments.

This series of stimulations gives an excellent opportunity to study the relation of auricular beats, maximal inhibition, and natural frequency. In 28 of the stimulations, three beats were required to cause maximal inhibition. In only one of the series was a different number required, in this case, four. This suggests that three beats is the number necessary to disperse the liberated acetylcholine to produce maximal inhibition when a stimulus of this strength is applied to the vagus. These three beats did not always occur within the same time interval but varied from 10 seconds to 16 seconds. This variance of time appears correlated with the natural frequency of the auricle. When the auricular rate was slow ("pericardial" solution), the time that it took for the three beats to occur was greater. That is, when the inhibition was the least,

the latent period for maximum inhibition was the greatest. This is contrary to what would be expected under the after-discharge theory. When the inhibition is decreased, whether due to a decreased strength of stimulation or to increased hydrolysis of acetylcholine, the delay period for maximum inhibition would also be expected to decrease because of the smaller amount of acetylcholine. Such was not the case; therefore the fact that it took longer for the three beats to occur with the least inhibition indicates that it is the beats that are responsible for the delay of maximum inhibition and not the release of more acetylcholine. If the delay for maximum inhibition were not dependent on the number of beats, the delay period could occur after any reasonable number of beats and not necessarily wait for just three to occur. It should be pointed out that three beats was the number required to produce maximum inhibition with this strength of stimulation to the vagus. A different strength or a different heart preparation might require more or less beats to produce maximum inhibition. The number required for any auricle to attain maximum inhibition at a specific strength of stimulation was consistent.

In a subsequent experiment, the chronotropic effect of multiple stimulation of the vagus nerve was studied. The stimulus used before the cholinesterase was applied was strong enough to completely inhibit the auricle for the period of stimulation (figure 10). Following this period of complete inhibition after the stimulus had been removed, the auricle began to recover. However, instead of the inotropic inhibition immediately starting to decrease, it actually continued to increase for 2-3 beats, lasting about 15 seconds. This increase did not occur if the auricle had been beating at all during the period that the stimulus was applied. Such a condition may be observed after the application of the cholinesterase (figure

10B). The chronotropic change was decreased and now, instead of getting complete inhibition, the auricle beat several times during the course of the vagal stimulation. Immediately following the period of stimulation the heart began its recovery phase. There was no longer any increase of the inotropic inhibition prior to the decrease. This evidence indicates that the latent period for the maximum inhibition with the single shock stimulation to the vagus is related to the beating of the auricle. When the auricle has been completely inhibited, the inotropic inhibition must still go through the latent increase in inhibition after the stimulation to the vagus is removed. It is the same type of delayed period that the single shock stimulation produced as is seen when compared with the single shock stimulus (figure 3) applied to the same auricular preparation. It suggests that the beat of the auricle actively forced the acetylcholine into parts of the auricle not reached when the auricle was initially inhibited. This experiment was specific evidence against the after-discharge of the postganglionic fibers. If after-discharge were to be assumed, there should be a proportionate amount of after-discharge following incomplete inhibition as there was following complete inhibition.

The evidence accumulated indicates that the beating of the auricle has an active part in the dispersion of the acetylcholine released by vagal stimulation. According to the Langmuir concept of heterogeneous reactions, this would serve to increase the surface area acted upon and in this preparation, increasing the surface area would increase the inhibition. The amount of inhibition obtained with a single shock would depend on the dispersion of the acetylcholine to a maximum by the beating of the auricle.

When the single shock stimulus was applied to the vagus, cholinesterase did not decrease the amount of inhibition but consistently

increased it by a slight amount. This occurred often enough to suggest that it was a significant item. The action of the cholinesterase has been clearly shown to decrease the amount of inotropic and chronotropic inhibition when multiple stimulation is applied to the vagus. Therefore it is safe to say that cholinesterase would act in the same manner when applied to the auricle being inhibited by a single shock. Why then, did slight increased inhibition consistently occur? Cholinesterase decreased the chronotropic inhibition, thus the rate of the auricular beats did not decrease as much as before the application of cholinesterase. If the rate does not decrease as much, the specific number of beats necessary to disperse the acetylcholine will occur sooner. There is less destuction of acetylcholine in this shorter time, the effect being to increase the auricular inhibition. Antagonistic to this action, the cholinesterase must have increased the rate of hydrolysis of acetylcholine tending to cause less inhibition with vagal stimulation. Therefore the amount of increase must be a balance between the decreased chronotropic effect which tends to increase the inhibition and the decreased inotropic inhibition due to the greater rate of hydrolysis. It appears that the decreased chronotropic effect which increases the inhibition is appreciably dominant. This, again, is not consonant with the after-discharge theory as an after-discharge would give less inhibition when cholinesterase was applied.

Following the application of any stimulus, the liberation of acetylcholine is stopped. There is probably no significant after-discharge for the reasons previously discussed. Therefore, there should be a specific amount of acetylcholine present in the auricle at the end of the stimulation, either single shock or multiple shocks. Figure 5B, 7B, 8B, and 9B were obtained by plotting the percent of inhibition against time for the recovery phase of each

stimulus for the experiments shown in figures 4 and 6. These show that the recovery of the auricle is due to a gradual decrease in the amount of inhibition until no inhibition exists. It is well established that the amount of inhibition that can be obtained by application of acetylcholine is proportional to the concentration applied. One may assume with ample justification that the percent of inhibition is proportional to the amount of acetylcholine present in the auricle at any specific time. Thus, the recovery curves should give the characteristics of the destruction of acetylcholine. The simple reaction of acetylcholine being hydrolyzed to acetate and choline is a first order reaction. The equation for a first order reaction is

$$\frac{dc}{dt} = K_1 c, \text{ and integrating,}$$

$$\ln c = K_1 t + K_2$$

$$\text{Log } c = K_1 t/2.303 + K_2$$

The logarithm of the percent inhibition (acetylcholine concentration) plotted against time (figure 12) should give a straight line. This plot was made for the recovery curves of figure 6B only, since all such recovery curves gave the same result. The straight line through these points was obtained by the least squares method applied to the logarithm of the percent inhibition. The slope of this line times 2.303 should give the velocity constant of the destruction of acetylcholine. When cholinesterase was applied to the auricle, the destruction rate increased. This is indicated by the increased slope of the straight line obtained after the cholinesterase was applied (figure 12). The velocity constant before the cholinesterase was 0.0345 and after the cholinesterase was 0.0470. When the cholinesterase was removed, the slope of the line nearly returned to the original control with a value of 0.0362.

Figure 12. Logarithm of the percent of inotropic inhibition plotted against time for the data shown in figure 6B.

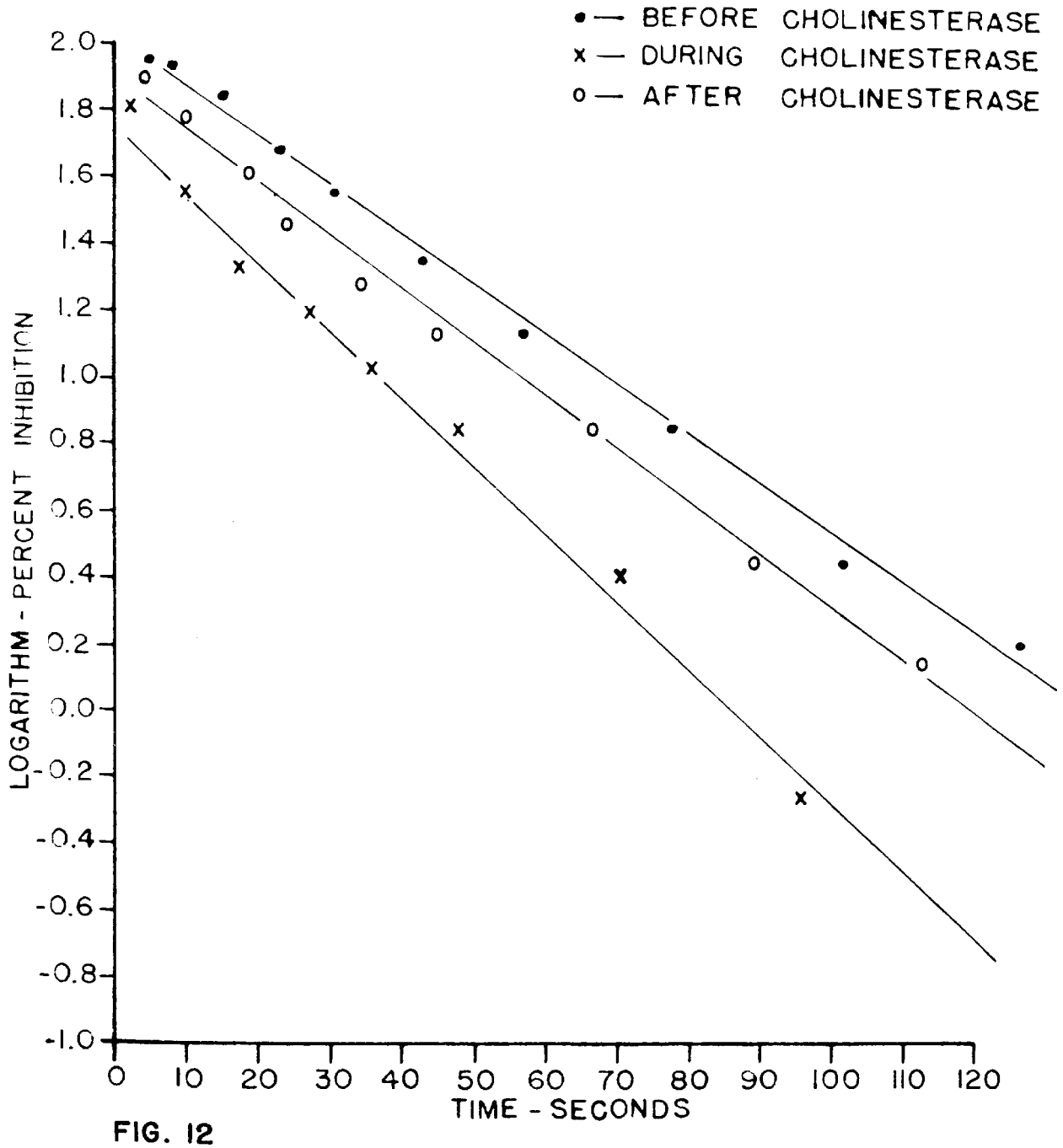

• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE
LOGARITHM - PERCENT INHIBITION
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
0
10
20
30
40
50
60
70
80
90
100
110
120
TIME - SECONDS

FIG. 12

As a further correlation of the equation for a first order reaction, the percent of inhibition per second was plotted against the average inhibition of the time measured (figure 13). In the two sets of data, before and after cholinesterase was applied, it is seen that the plotted points give a relatively straight line after the first three or four beats. The straight line indicates that the rate of destruction of acetylcholine is proportional to the concentration as is required by the equation of a first order reaction. The first three or four beats are required to disperse the liberated acetylcholine until a homogeneous destruction occurs. When the cholinesterase was applied, a line with a greater slope was obtained, indicating that the rate of hydrolysis has increased. Also noted, it did not require the three or four beats before equilibrium was established since the strength of the beat was sufficient to disperse the acetylcholine to a maximum with the decreased inhibition due to cholinesterase. This evidence suggests that the beating of the auricle serves to spread the liberated acetylcholine.

Summary

It was found that the amount of inhibition of the auricle from a single shock of a constant strength to the vagus nerve depended on the perfusing solution. The solution containing the most bicarbonate resulted in the least inhibition.

The inhibition of the auricle due to vagal stimulation may be divided into inotropic and chronotropic inhibition. Cholinesterase applied to the auricle decreased both types of inhibition if multiple shock stimulation was applied to the vagus. If single shocks were used, cholinesterase tended to increase the inotropic effects.

The manner in which the acetylcholine accumulated from each successive shock of a multiple shock stimulation was postulated mathe-

Figure 13. Rate curve. Percent inhibition per second plotted against average percent inhibition for the data shown in figure 6B.

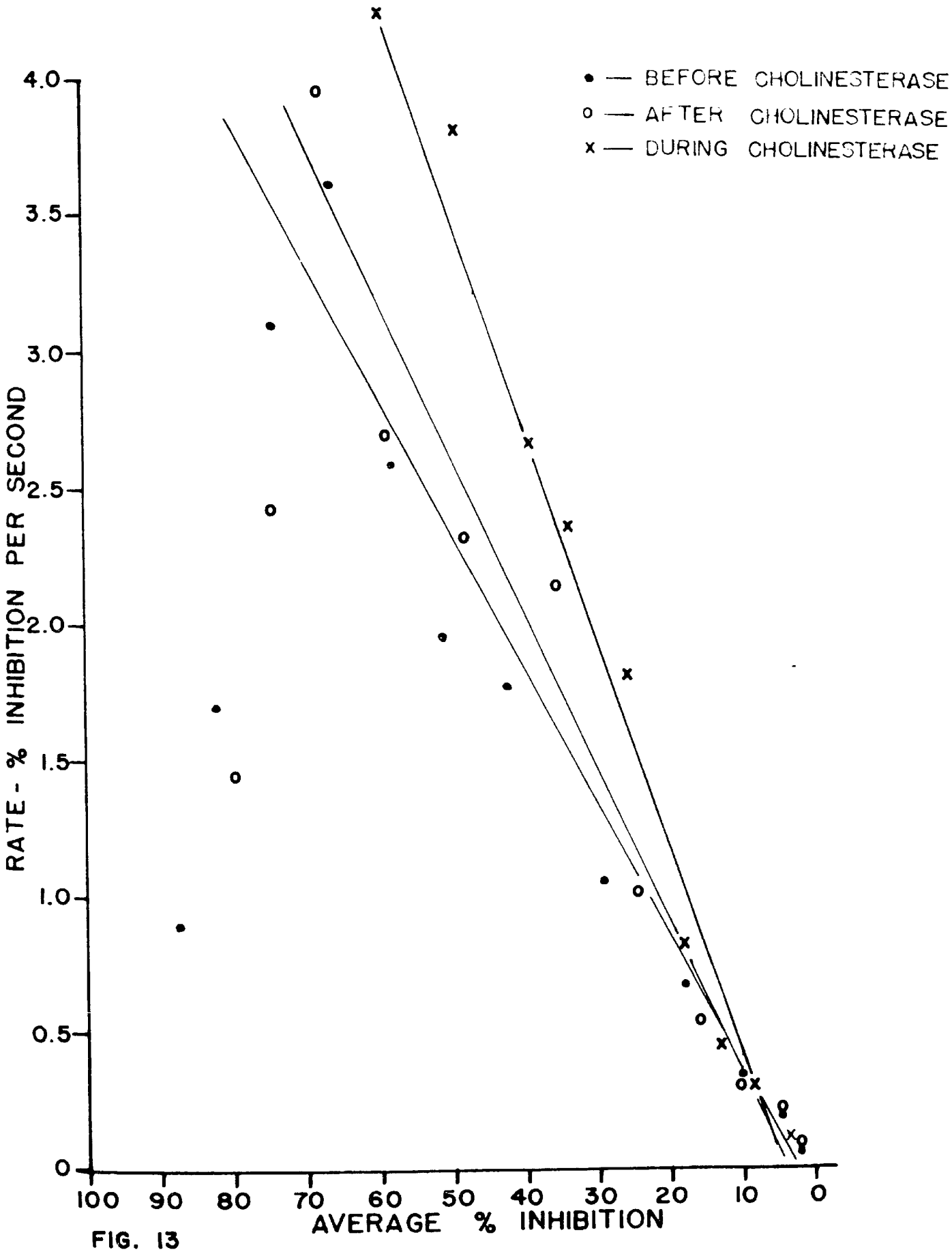

• — BEFORE CHOLINESTERASE
o — AFTER CHOLINESTERASE
x — DURING CHOLINESTERASE
RATE - % INHIBITION PER SECOND
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
100 90 80 70 60 50 40 30 20 10 0
AVERAGE % INHIBITION

FIG. 13

matically. The resulting curves fit the experimental data closely.

It was suggested that the inhibition due to vagal stimulation may follow Langmuir's theory of heterogeneous reactions. On the basis of this postulate, the amount of inhibition would be a function of the area available to the acetylcholine and a function of the concentration of acetylcholine. The area available to acetylcholine depends on the strength of stimulation and on the diffusion of the acetylcholine, diffusion due to concentration gradients and diffusion due to the force applied by the beating of the auricle.

It was shown in several ways that the beat of the auricle must be a contributing factor. A. When partial inhibition occurred due to a single shock stimulation,the maximum inhibition does not occur immediately, but only after several beats. Existing evidence indicates that the time required for maximum inhibition to occur is too long to be explained on the after-discharge theory. Some other factor is indicated as causing the delay of maximum inhibition.

B. When different types of salt solutions were used, different degrees of inhibition were obtained using the same strength of stimulation to the vagus nerve. The number of beats that occurred before the advent of maximum inhibition was the same regardless of the degree of inhibition, whereas the time that it took the beats to occur was not the same. Since the same number of beats occurred in different periods of time, it is reasonable to suspect that the beats are concerned with the delay of maximum inhibition.

C. After the cessation of a vagal stimulation which caused complete inhibition of the auricle, the inotropic inhibition continued to increase for a period of time similar to the single shock inhibition. After cholinesterase was added to the preparation and complete inhibition did not occur, the inotropic inhibition did not increase before decreasing. Thus, it appears that the beats which

occur during stimulation affect the subsequent recovery curves.

D. When the cholinesterase was applied to an auricle and the vagus stimulated with a single shock, the amount of inhibition increased when compared with the control period before and after cholinesterase. If the delay of maximum inhibition were due only to after-discharge, cholinesterase would have to cause a decrease in the inhibition. If the delay were assigned to the beating of the auricle, the increase of inhibition was explainable. This represents indirect evidence for the effect of the beating of the auricle on the dispersion of the acetylcholine.

If one considers the amount of inhibition proportional to the amount of acetylcholine present in the auricle, the recovery curves represent the characteristics of the reaction that removes acetylcholine. This follows a first order reaction. When cholinesterase was applied, a first order reaction was obtained with a greater velocity constant. This is what was expected from the action of cholinesterase on the rate of acetylcholine destruction.

CHORDA TYMPANI-SUBMAXILLARY GLAND SYSTEM

Method

The submaxillary gland and its parasympathetic nerve supply (chorda tympani nerve) were exposed in the conventional manner in the dog. Salivary flow from Wharton's duct was measured by an electronic drop recorder. All branches and tributaries of the carotid arteries and external jugular vein respectively, except those to and from the submaxillary gland were ligated. Another drop recorder was connected to the external jugular vein to measure blood flow through the gland. Platinum electrodes from an electronic square-wave stimulator were placed on the chorda tympani and were not moved throughout any procedure. Cholinesterase was injected into the common carotid artery.

Results and Discussion

The parasympathetic control of the submaxillary gland is mediated through the chorda tympani nerve, a branch of the seventh cranial nerve. The general visceral efferent fibers of the chorda tympani synapse in a ganglion distributed along the entire pathway from the chorda lingual triangle well into the gland. These are the postganglionic cells of the parasympathetic system and are well supplied with blood vessels. (Langley, 1890; Gray, 1942; Bradley, 1948)

Gibbs and Szeloczey (1932) were the first to show that the chorda tympani stimulation caused the appearance of an acetylcholine-like substance, "Chordastoff", in the solution perfusing from the submaxillary gland. The action of the acetylcholine liberated by the cholinergic chorda tympani is a function of the time that it takes to destroy the acetylcholine. Cholinesterase applied to the system will destroy the acetylcholine faster and diminish the response of the organ.

Stimulation of the cut peripheral end of the chorda tympani caused two measureable effects, an increase in salivary flow and an increase in blood flow through the gland (figure 14). The increase in blood flow is caused by the dilation of blood vessels within the gland.

Salivary flow in a normal preparation was discontinuous; that is, it did not occur unless the chorda tympani was stimulated. On stimulation with a constant tetanic stimulus applied for a definite period, the submaxillary gland secreted a specific amount of saliva. In this preparation the amount of saliva was measured by recording the drops of saliva that occurred with each stimulation. Calibration of the volume of the drops of saliva showed that each drop contained 0.0456 cc. or that there were 21.8 drops in each cc. of saliva collected.

The blood flow through the gland was not discrete as was the salivary flow, but rather, there was a continual flow. Before any stimulus was applied to the chorda tympani nerve, the continual blood flow was checked to see if it appeared in the normal range for that particular animal. In the experiment shown, the normal blood flow ranged from 10 to 12 drops of blood per minute for the entire experiment. This is significant and will be considered later. Calibration of the volume of the drops of blood showed that each drop contained 0.0802 cc. or there were 12.5 drops in each cc. of blood collected.

Cholinesterase was injected into the carotid artery and allowed to pass only to the isolated submaxillary gland. An attempt was made to prevent any other physiological process being affected directly by the cholinesterase. The method also provided a means of applying a concentrated amount of cholinesterase to the gland since it was found that in small amounts, the cholinesterase had

Figure 14. Experiment to show the effect of cholinesterase on recorded salivary flow and blood flow of the submaxillary gland when the chorda tympani was stimulated.

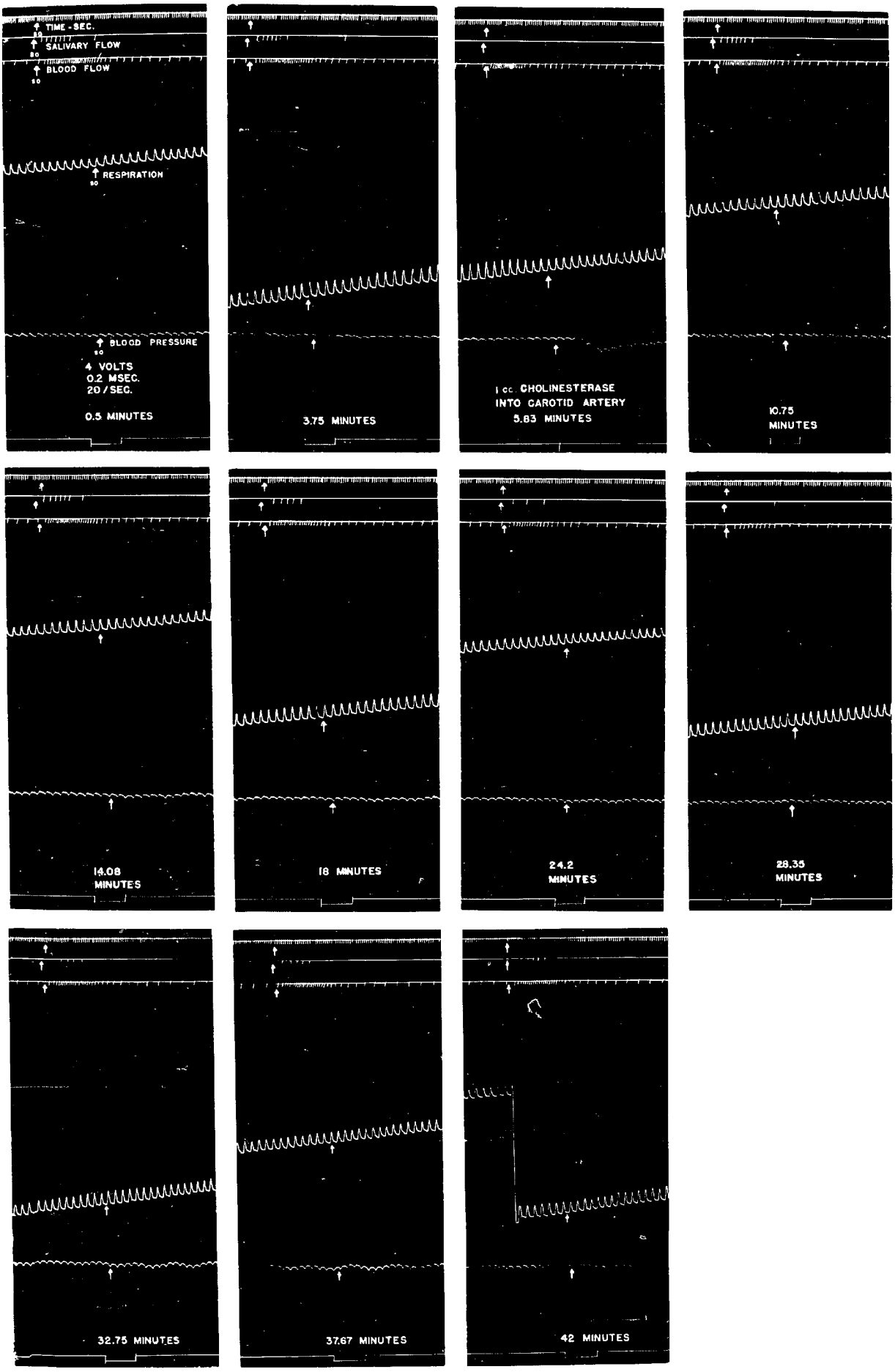

TIME - SEC.
10
SALIVARY FLOW
BLOOD FLOW
RESPIRATION
BLOOD PRESSURE
4 VOLTS
0.2 MSEC.
20 / SEC.
0.5 MINUTES
3.75 MINUTES
1 cc. CHOLINESTERASE
INTO CAROTID ARTERY
5.83 MINUTES
10.75
MINUTES
14.08
MINUTES
18 MINUTES
24.2
MINUTES
28.35
MINUTES
32.75 MINUTES
37.67 MINUTES
42 MINUTES

FIG. 14

little or no discernable effect. This was probably because the cholinesterase had to leave the blood stream to have maximal action at the nerve endings. Since the cholinesterase molecule is estimated as having a molecular weight of approximately 3 million (Nachmansohm, 1948), movement through the blood vessel wall would probably only take place if there were a large gradient. This gradient was obtained by concentration of the entire injection focused on the submaxillary gland.

After injection of the cholinesterase, chorda tympani stimulation caused less increase in both the saliva flow and the blood flow. The saliva flow changed from eight drops per 15 second stimulation to one drop 22 minutes after the injection of the cholinesterase. This is a decrease of 87.5 percent in the salivary flow. The time course for the drops of saliva is plotted for the stimulation immediately before the injection of cholinesterase, for the stimulation showing the maximal effect of cholinesterase, and for the stimulation after the recovery from cholinesterase (figure 15). The secretion began almost immediately after the stimulus was applied. Within a few seconds the secretion attained a constant rate that prevailed throughout the fifteen second stimulation. At the end of the stimulation the rate of secretion gradually decreased until no more drops were formed. The time that it took the last drop to be released does not appear to have any correlation with the number of drops formed for any stimulation in these experiments. However, this can probably be attributed to the mechanical insufficiencies of the biological system and the recording system.

The increased blood flow through the submaxillary gland with chorda tympani stimulation should be the resultant of several factors. The number of blood vessels being affected is a factor that depends on the strength of stimulation. The length of the stimulation

Figure 15. Salivary flow from the submaxillary gland when the chorda tympani was stimulated before cholinesterase, during maximal cholinesterase effect, and after recovery from the injected cholinesterase.

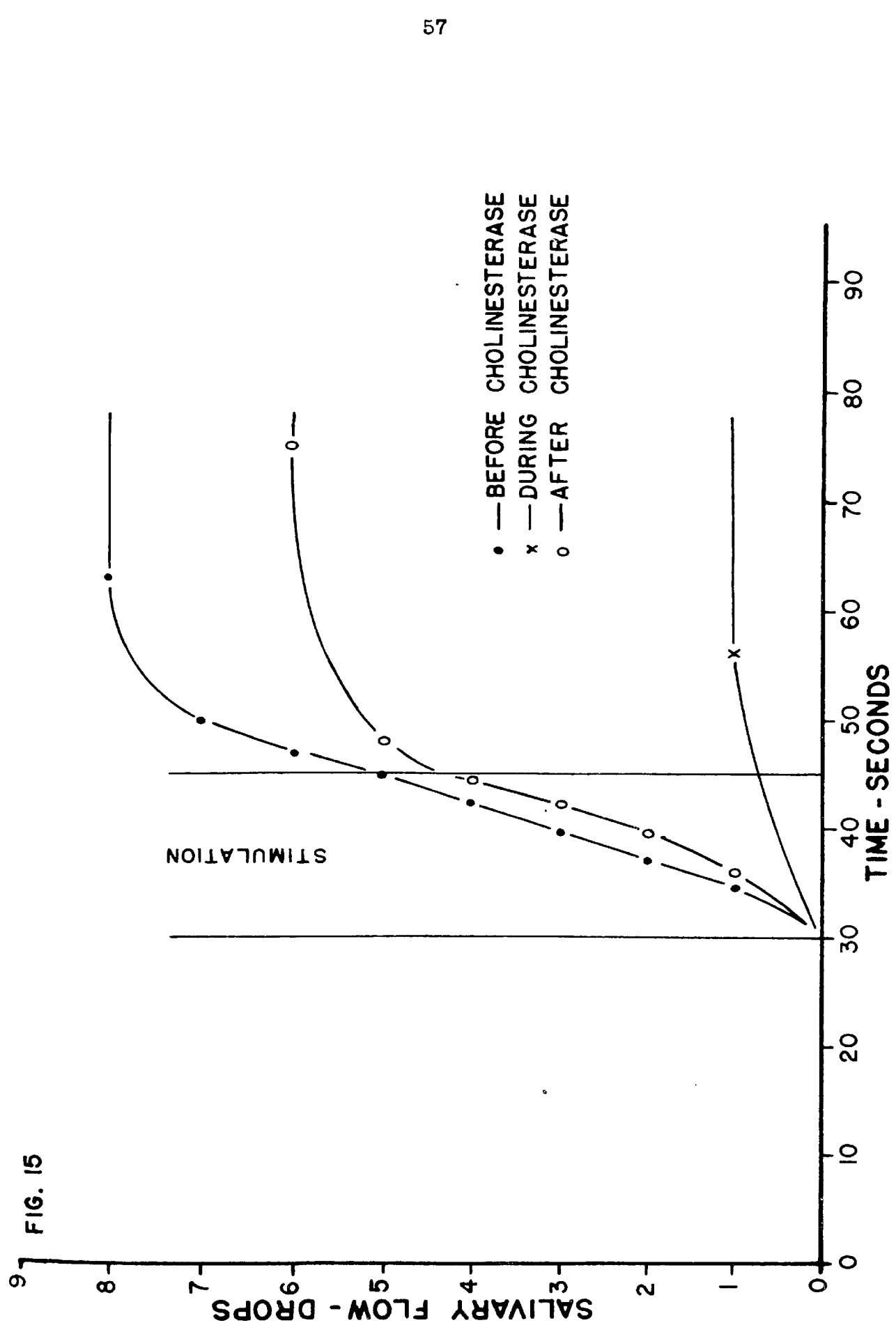

SALIVARY FLOW - DROPS
TIME - SECONDS
STIMULATION
• —BEFORE CHOLINESTERASE
x —DURING CHOLINESTERASE
o —AFTER CHOLINESTERASE

FIG. 15

would also be a factor influencing the increase in blood flow by maintaining a sustained release of acetylcholine. Greater frequency of stimulation increases the rate of release of acetylcholine, thus allowing an increased accumulation. All of these factors have been kept constant in the experiment and therefore are not considered.

The only factor varied in the experiment was the rate of destruction of acetylcholine and this was changed by the injection of cholinesterase. The blood flow through the gland was continual, thus the increased blood flow due to chorda tympani stimulation must necessarily be adjusted to determine the true increase. This was most easily done by plotting the drops of blood against time (figure 16) for the period before cholinesterase, the period of maximal cholinesterase activity, and the period after the effect of cholinesterase had diminished. Then straight lines were drawn through the points before the stimulation and after the stimulation. Since these lines are obviously straight and since the lines drawn before and after the stimulation for each of the three periods are parallel, it can be stated that the rate of normal blood flow was constant. The perpendicular distance between the line drawn for the interval before any stimulus was applied and after the stimulus was stopped is an indication of the increase of blood flow due to the chorda tympani stimulation.

The blood flow increased by 24 drops during the control chorda tympani stimulation from the time the stimulus was started until normal blood flow was again recorded. During the maximal cholinesterase effect, chorda tympani stimulation caused an increase of 6.8 drops. This is a decrease of 71.1 percent of the control stimulation. After the maximal effect of the cholinesterase, blood flow due to chorda tympani stimulation gradually returned to an increase of 20 drops, but did not entirely recover.

Figure 16. Blood flow through the submaxillary gland with chorda tympani stimulation before cholinesterase, during maximal cholinesterase effect, and after recovery from the injected cholinesterase.

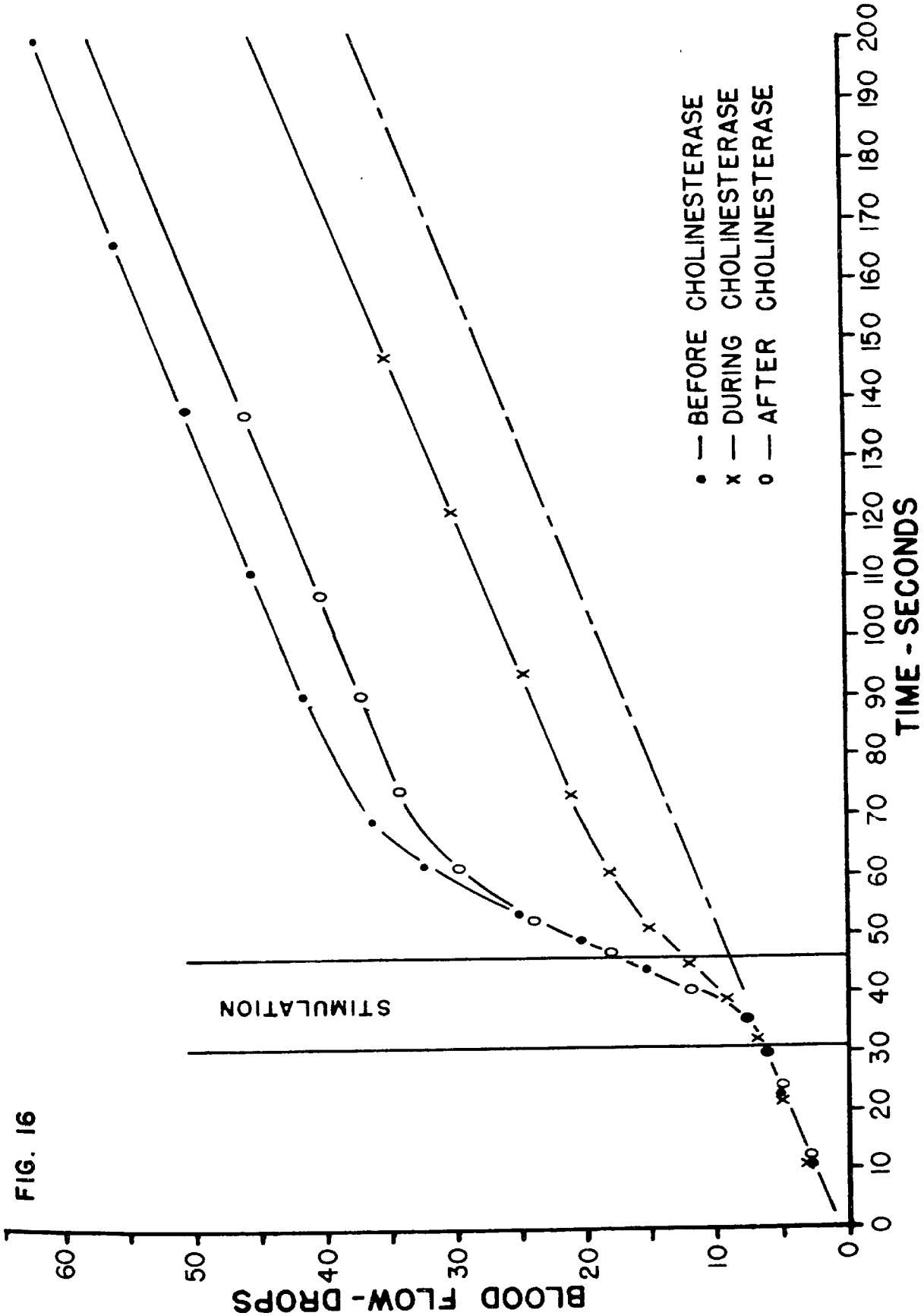

BLOOD FLOW-DROPS
TIME-SECONDS
STIMULATION
• — BEFORE CHOLINESTERASE
x — DURING CHOLINESTERASE
o — AFTER CHOLINESTERASE
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200

FIG. 16

There are two possible reasons why the blood flow did not completely return to the original control value of 24 drops. It is conceivable that some of the cholinesterase remained in the tissue. The second reason involves the normal blood flow through the gland. The factors controlling the normal blood flow are the blood pressure and the cross-sectional area of the blood vessels of the gland. If the blood pressure decreases, the blood flow should also decrease unless counteracted by an increase in the cross-sectional area of the blood vessels. The record of the blood pressure indicate that at the beginning of the experiment the blood pressure was 106 mm. of mercury. After the cholinesterase effect had diminished and the amount of blood flow had increased to 20 drops per chorda tympani stimulation, the blood pressure was 92 mm. of mercury. The normal blood flow was still within the 10-12 drops per minute. This means that the cross-sectional area of the blood vessels must be greater than at the beginning of the experiment to maintain this constant flow. If this is true, then the effect of a chorda tympani stimulation may not be as great because the blood vessels are already partially dilated or because some blood vessels are open that were not open previously.

Summary

The effect of a cholinesterase preparation was studied by injecting it into the isolated blood supply of the submaxillary gland. Increased blood flow and increased salivary flow, produced by chorda tympani stimulation, were used as criteria of the cholinesterase activity. Cholinesterase diminished the increased salivary flow and the increased blood flow by increasing the rate of hydrolysis of the acetylcholine liberated by chorda tympani stimulation.

SUPERIOR CERVICAL GANGLION

Method

Isotonic contractions of the nictitating membrane, recording on a kymograph, were used as an index of superior cervical ganglion activity when the preganglionic nerve was stimulated. Cholinesterase was either applied topically or was injected using a modification of Kibjakow's method (1933). The preganglionic nerve was placed in a reversible fluid-bridge electrode connected to an electronic square-wave stimulator. (see appendix)

Results

The superior cervical ganglion was studied by stimulating the preganglionic nerve and recording the resulting contraction of the nictitating membrane. If the strength of the stimulation at a low frequency (2-20 shocks per second usually) is increased, the height of contraction increases until it reaches a maximum. A supermaximal stimulus has no further effect. If the frequency of stimulation is increased at a constant strength, the maximal height of contraction decreases and there is a subsequent decline after the new maximum has been attained. The subsequent decline depends on the frequency of stimulation; the higher the frequency, the greater will be the decrease. This is by no means a new phenomenon, as Orías (1932) studied it, concluding that it was due to a Wedensky inhibition. Rosenblueth, et al, (1936) supported Orías' results.

Topical application may be illustrated by the typical experiment shown in figure 17. There were five frequencies of stimulation, 5, 10, 20, 30, and 50 shocks per second, applied to the preganglionic cervical sympathetic. These were repeated every 15 minutes with 2.5 minute intervals between each frequency of stimulation. A control was established, then cholinesterase was applied to the

Figure 17. Topical application of cholinesterase to the superior cervical ganglion recording the contractions of the nictitating membrane due to five different preganglionic stimuli.

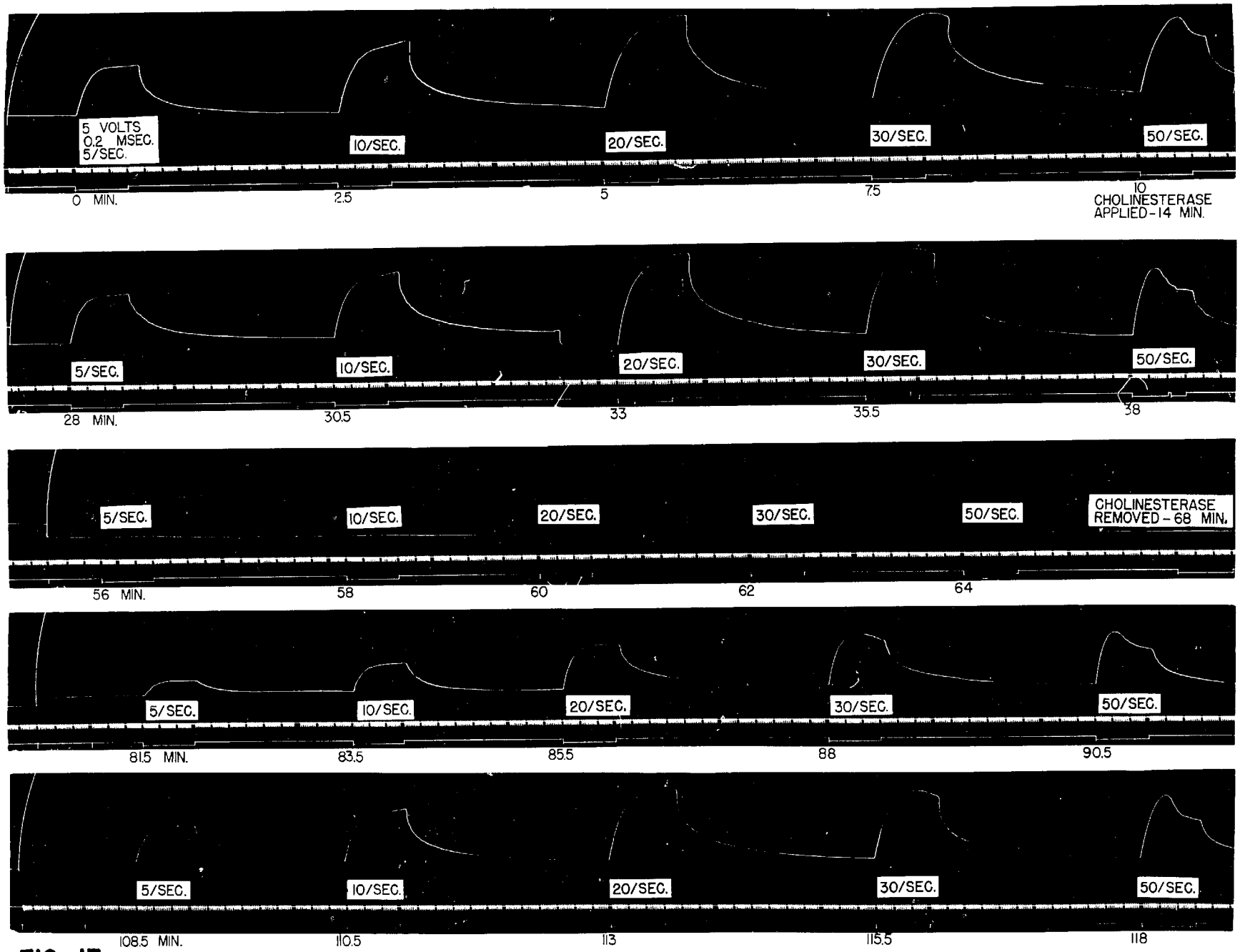

5 VOLTS
0.2 MSEC.
5/SEC.
10/SEC.
20/SEC.
30/SEC.
50/SEC.
0 MIN.
2.5
5
7.5
10
CHOLINESTERASE
APPLIED-14 MIN.
5/SEC.
10/SEC.
20/SEC.
30/SEC.
50/SEC.
28 MIN.
30.5
33
35.5
38
5/SEC.
10/SEC.
20/SEC.
30/SEC.
50/SEC.
CHOLINESTERASE
REMOVED-68 MIN.
56 MIN.
58
60
62
64
5/SEC.
10/SEC.
20/SEC.
30/SEC.
50/SEC.
81.5 MIN.
83.5
85.5
88
90.5
5/SEC.
10/SEC.
20/SEC.
30/SEC.
50/SEC.
108.5 MIN.
110.5
113
115.5
118

FIG. 17

ganglion. The cholinesterase was allowed to remain on the ganglion until there was a definite change in the contraction of the nictitating membrane. In the experiment presented, recordable contractions ceased 42 minutes after the application of cholinesterase. Following this series of stimulations, the cholinesterase was removed and the ganglion bathed with warm 0.9 percent saline solution until a stable series of contractions was recorded. The height of the contraction at the five frequencies was plotted against time (figure 18). This shows the progressive nature of the cholinesterase effect and the subsequent recovery.

Another experiment is reported in which the effect of cholinesterase did not attain a 100 percent decrease in the height of contraction (figure 19). The cholinesterase was applied immediately after the control stimulations of 10, 20, 30, and 50 shocks per second to the preganglion nerve. The height of the contraction was plotted against time for each of the frequencies, before, during, and after the cholinesterase (figure 20). When the cholinesterase was applied, the amount of contraction for each of the frequencies gradually decreased. After 55 minutes, the cholinesterase was replaced with 0.9 percent saline and the observations continued for about 100 minutes. Complete recovery was not achieved even though the ganglion was repeatedly irrigated with fresh saline solution.

A vertical line was drawn on the graph (figure 20) at 53 minutes and the interpolated values obtained for each of the frequencies. The cholinesterase caused the greatest change when the higher frequencies of stimulation were used (Table 3).

Figure 18. Contraction height of the nictitating membrane plotted against time for the experiment shown in figure 17.

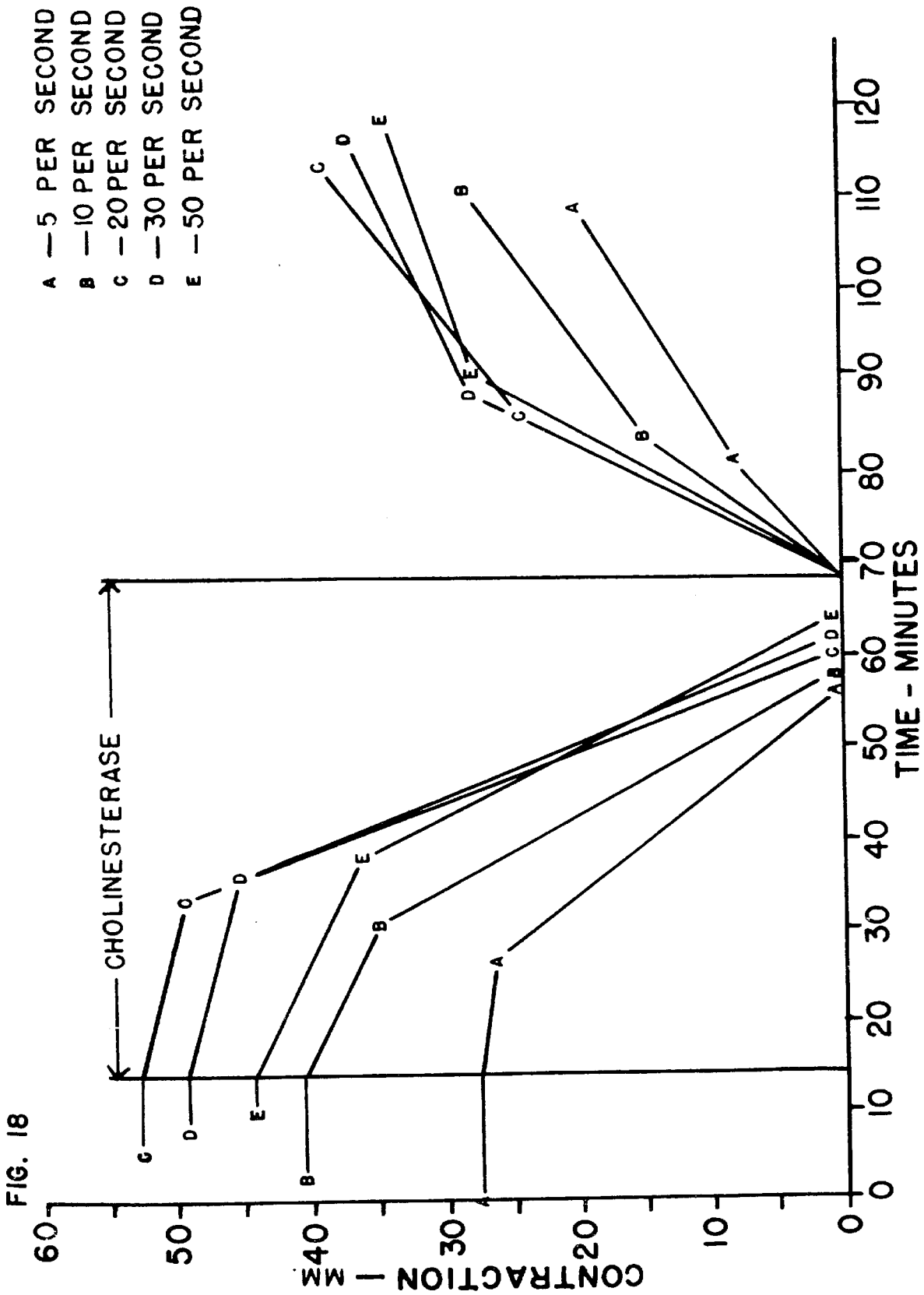

A —5 PER SECOND
B —10 PER SECOND
C —20 PER SECOND
D —30 PER SECOND
E —50 PER SECOND
CHOLINESTERASE
CONTRACTION — MM
TIME - MINUTES
0
10
20
30
40
50
60
70
80
90
100
110
120

FIG. 18

Figure 19. Topical application of cholinesterase to the superior cervical ganglion recording the contractions of the nictitating membrane from four different preganglionic stimuli.

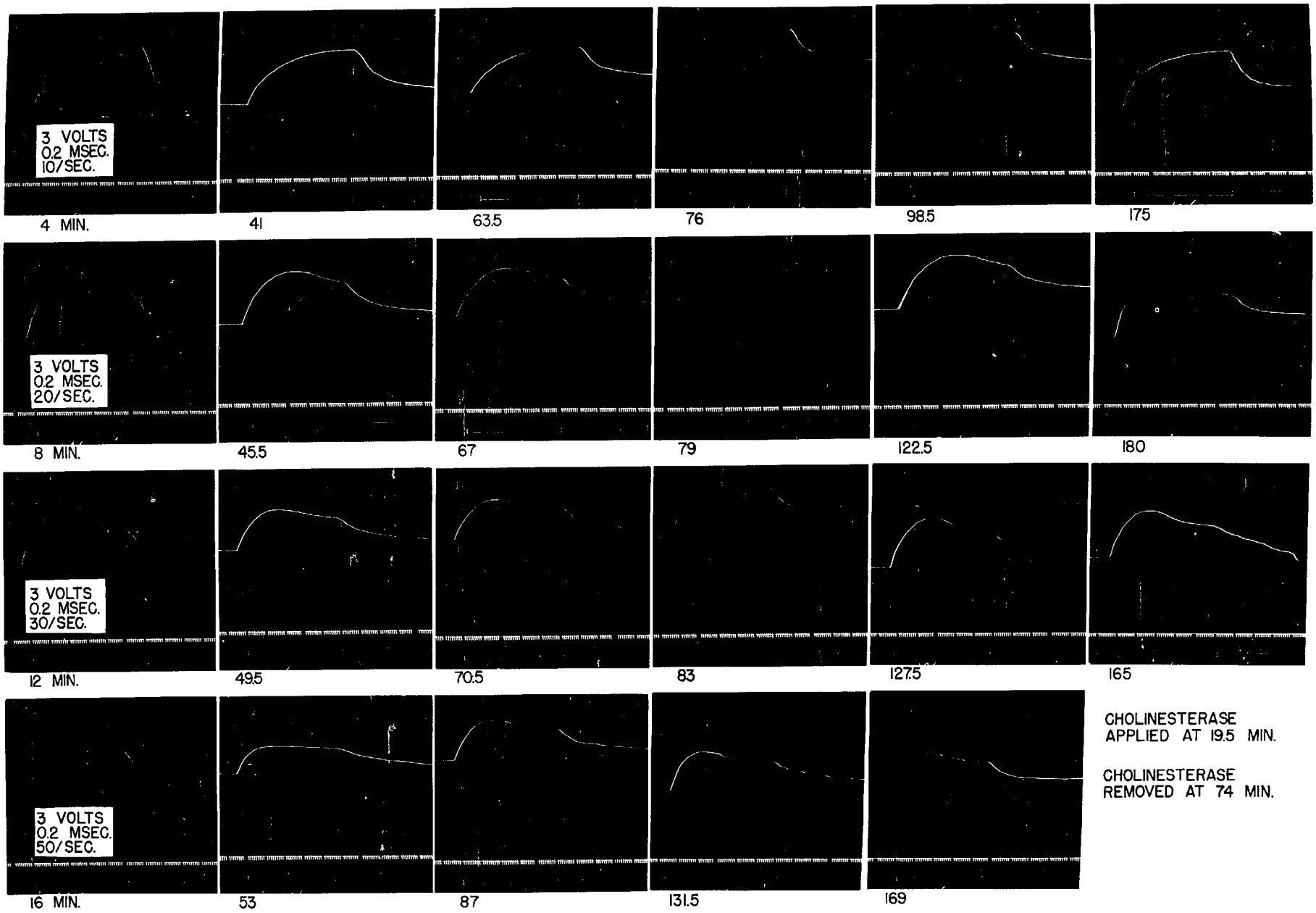

3 VOLTS
0.2 MSEC.
10/SEC.
4 MIN.
41
63.5
76
98.5
175
3 VOLTS
0.2 MSEC.
20/SEC.
8 MIN.
45.5
67
79
122.5
180
3 VOLTS
0.2 MSEC.
30/SEC.
12 MIN.
49.5
70.5
83
127.5
165
3 VOLTS
0.2 MSEC.
50/SEC.
16 MIN.
53
87
131.5
169
CHOLINESTERASE
APPLIED AT 19.5 MIN.
CHOLINESTERASE
REMOVED AT 74 MIN.

FIG. 19

Figure 20. Contraction height of the nictitating membrane plotted against time for the experiment shown in figure 19.

FIG. 20

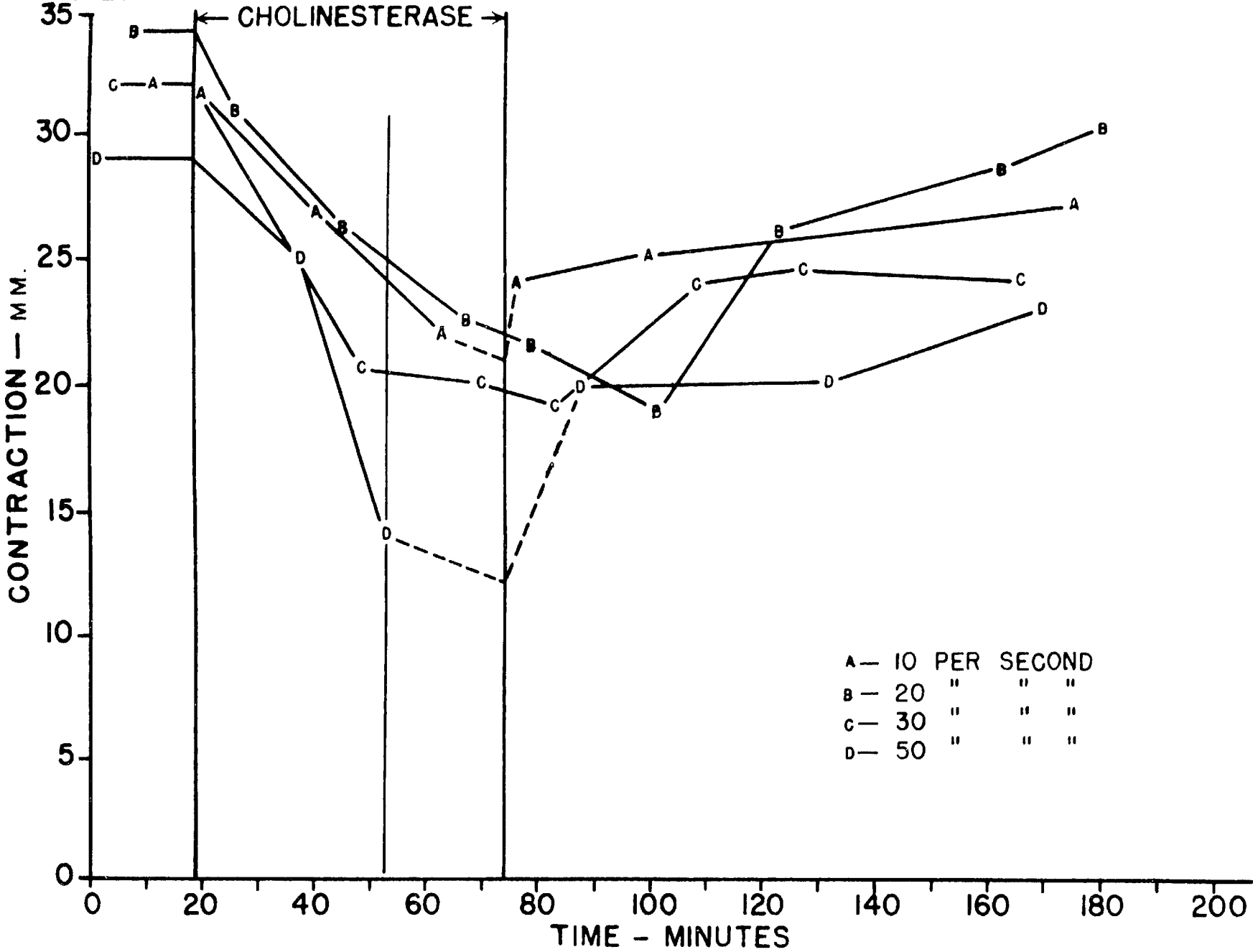

← CHOLINESTERASE →
CONTRACTION — MM.
0
5
10
15
20
25
30
35
0
20
40
60
80
100
120
140
160
180
200
TIME - MINUTES
A— 10 PER SECOND
B— 20 " " "
C— 30 " " "
D— 50 " " "

TABLE 3

Frequency of stimulation	Height of contraction - mm. Before cholinesterase	After cholinesterase, interpolated	Percent decrease
10	32	24.2	24.4
20	34	24.7	27.4
30	32	20.3	36.6
50	29	14.0	51.8

When the cholinesterase was injected into the circulatory system, the effect was not as conclusive as topical application. Also, the number of different frequencies that could be studied during one application of cholinesterase was limited by the time of perfusion through the ganglion. In one such experiment, the same stimulation was applied at alternately 2 and 5 minute intervals (figure 21). The strength and the frequency of stimulation were previously determined as the maximum that would cause a sustained contraction. The height of contraction of the stimulation A before the cholinesterase was 36.7 mm. The greatest decrease occurred 16 minutes after the injection when the contraction was 31.0 mm. This is a 15.5 percent decrease in the height of contraction. Twenty-nine minutes after the injection the contraction returned to 33.4 mm.

The second stimulation, B, caused a contraction of 34.8 mm. before the cholinesterase was injected. Thirty-one minutes after the cholinesterase, the contraction was 29.2 mm. This is a 16.2 percent decrease. The contraction returned to a height of 31.1 mm. in 31 minutes. In neither case did the height of contraction return to the original level.

A second experiment showing the effect of injected cholinesterase was selected in which the frequency of stimulation was sufficient to cause a perceptible decrease following the initial

Figure 21. Contractions of the nictitating membrane due to stimulation of the preganglionic nerve when cholinesterase was injected into the circulatory system of the superior cervical ganglion.

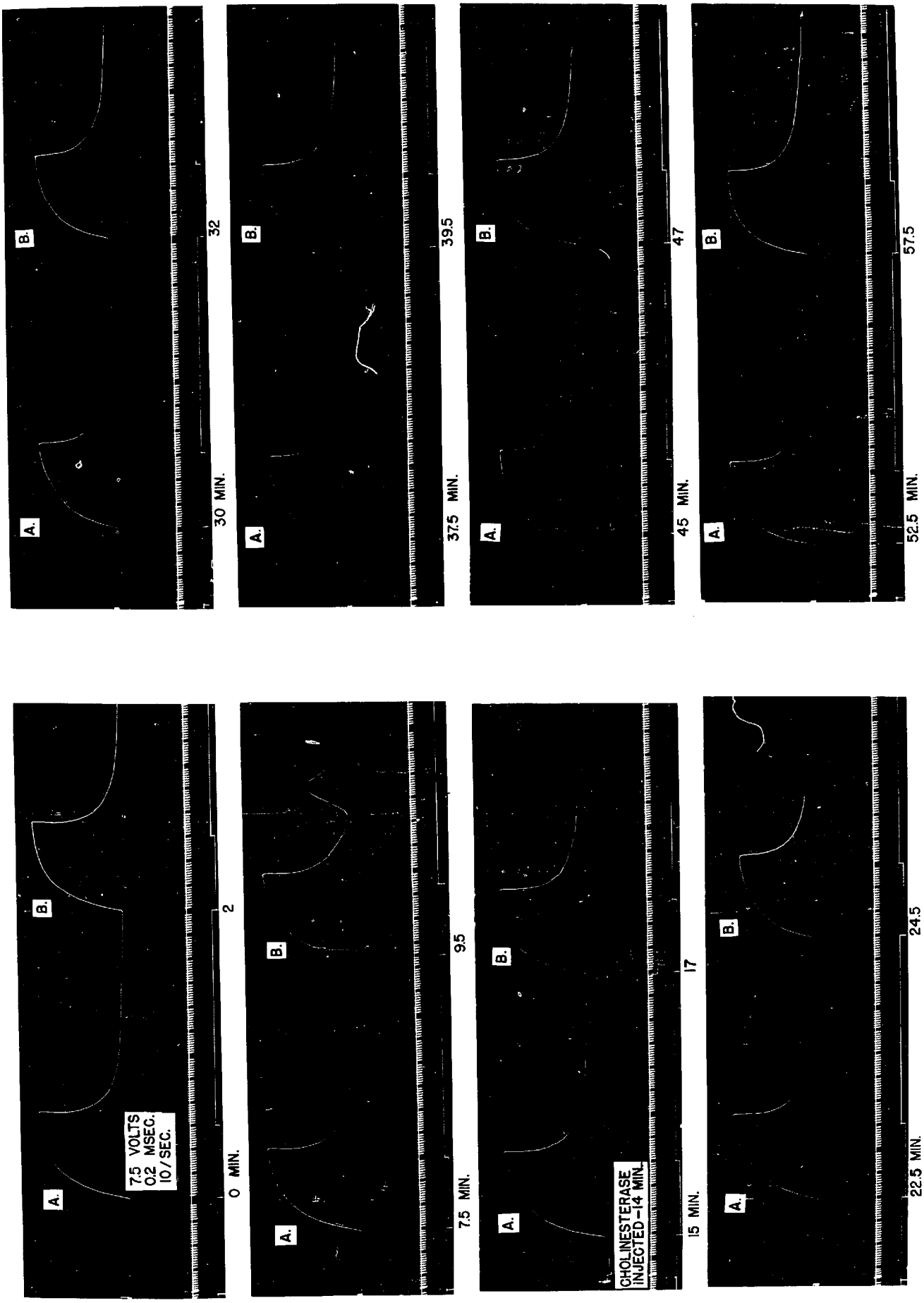

A.
B.
7.5 VOLTS
0.2 MSEC.
10/SEC.
0 MIN.
2
7.5 MIN.
9.5
15 MIN.
17
22.5 MIN.
24.5
CHOLINESTERASE
INJECTED-14 MIN.
30 MIN.
32
37.5 MIN.
39.5
45 MIN.
47
52.5 MIN.
57.5

FIG. 21

maximal response (figure 22). The maximal height of contraction was plotted against time to give the time course of the action of the cholinesterase (figure 23). Controls were established and then cholinesterase was injected. The height of contraction gradually decreased 43.8 percent, going from 17.8 mm. to approximately 10 mm., 22 minutes after the application of cholinesterase. The nicitating membrane remained at this level for about 25 minutes and then gradually increased to 16 mm.

Discussion

That the acetylcholine-cholinesterase system plays an integral part in synaptic transmission has been demonstrated by a succession of discerning experiments. First Kibjakow (1933), then Feldberg and Gaddum (1934) showed that when the preganglionic nerve to the superior cervical ganglion was stimulated, the perfusing solution going through the isolated circulatory system of the ganglion contained acetylcholine. Gesell's theory of neurocellular generation suggests that this acetylcholine, liberated by preganglionic fibers, increases the neurocellular current. The amount of neurocellular current will determine the firing of the postganglionic axon. Since Larrabee and Bronk (1938) found that there is no after-discharge with stimulations below 20 per second to the nerve, the acetylcholine must be destroyed very rapidly to prevent its persistent action on the postganglionic cell. This necessitates a high concentration of cholinesterase in the area of the release of the acetylcholine to facilitate the required rapid destruction. Nachmansohm (1940) concluded that the amount of cholinesterase present was sufficient to destroy the amount of acetylcholine liberated with a single shock stimulus to the preganglionic nerve within a supposed refractory period of 2 milliseconds. Even though Gesell's theory does not require a refractory period, Nachmansohm's investigations

Figure 22. Contractions of the nictitating membrane from stimulation of the preganglionic nerve when cholinestesterase was injected into the circulatory system of the superior cervical ganglion.

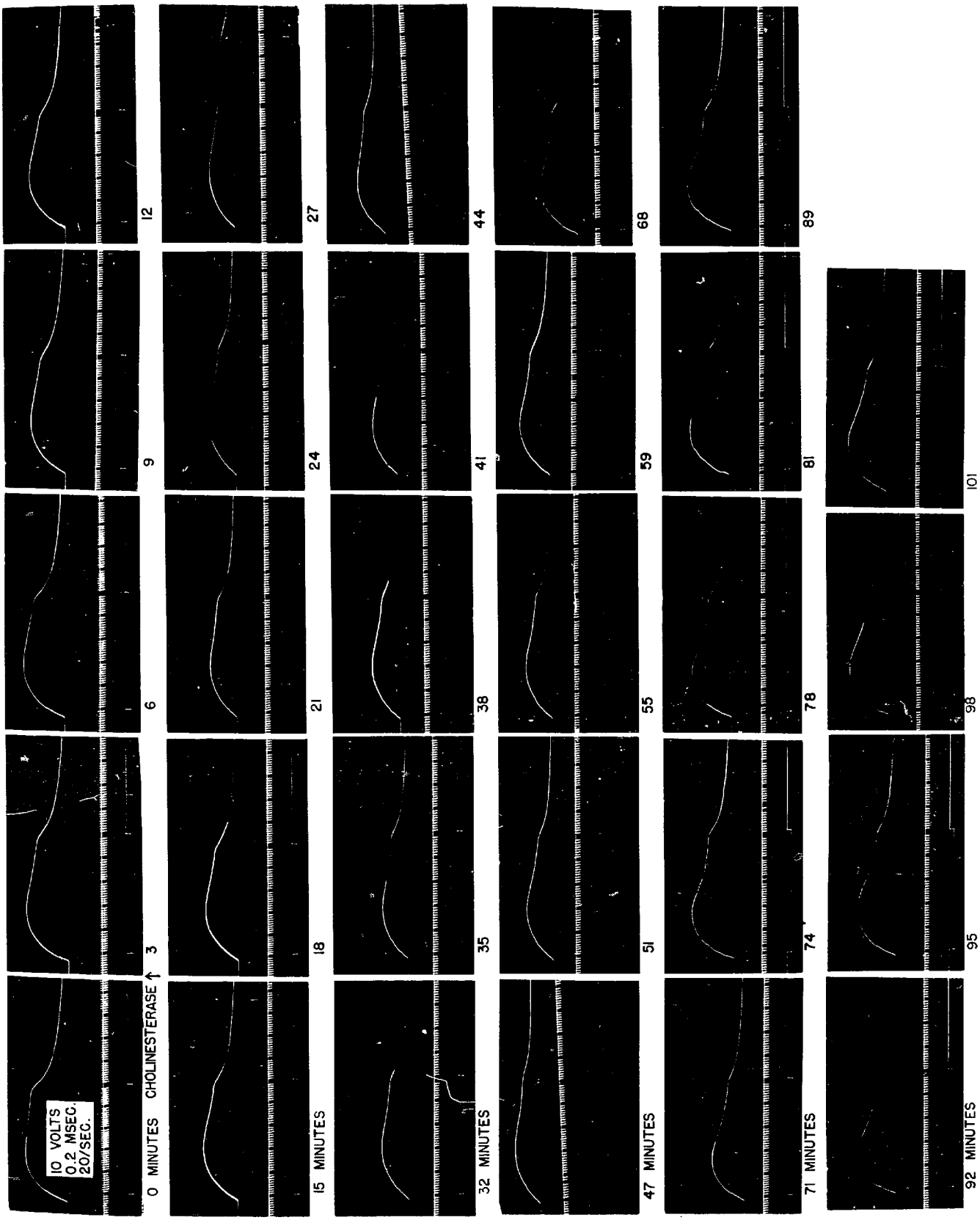

10 VOLTS
0.2 MSEC.
20/SEC.
0 MINUTES CHOLINESTERASE
3
6
9
12
15 MINUTES
18
21
24
27
32 MINUTES
35
38
41
44
47 MINUTES
51
55
59
68
71 MINUTES
74
78
81
89
92 MINUTES
95
98
101

FIG. 22

Figure 23. Contraction height for each stimulation of the nictitating membrane plotted against time for the experiment shown in figure 22.

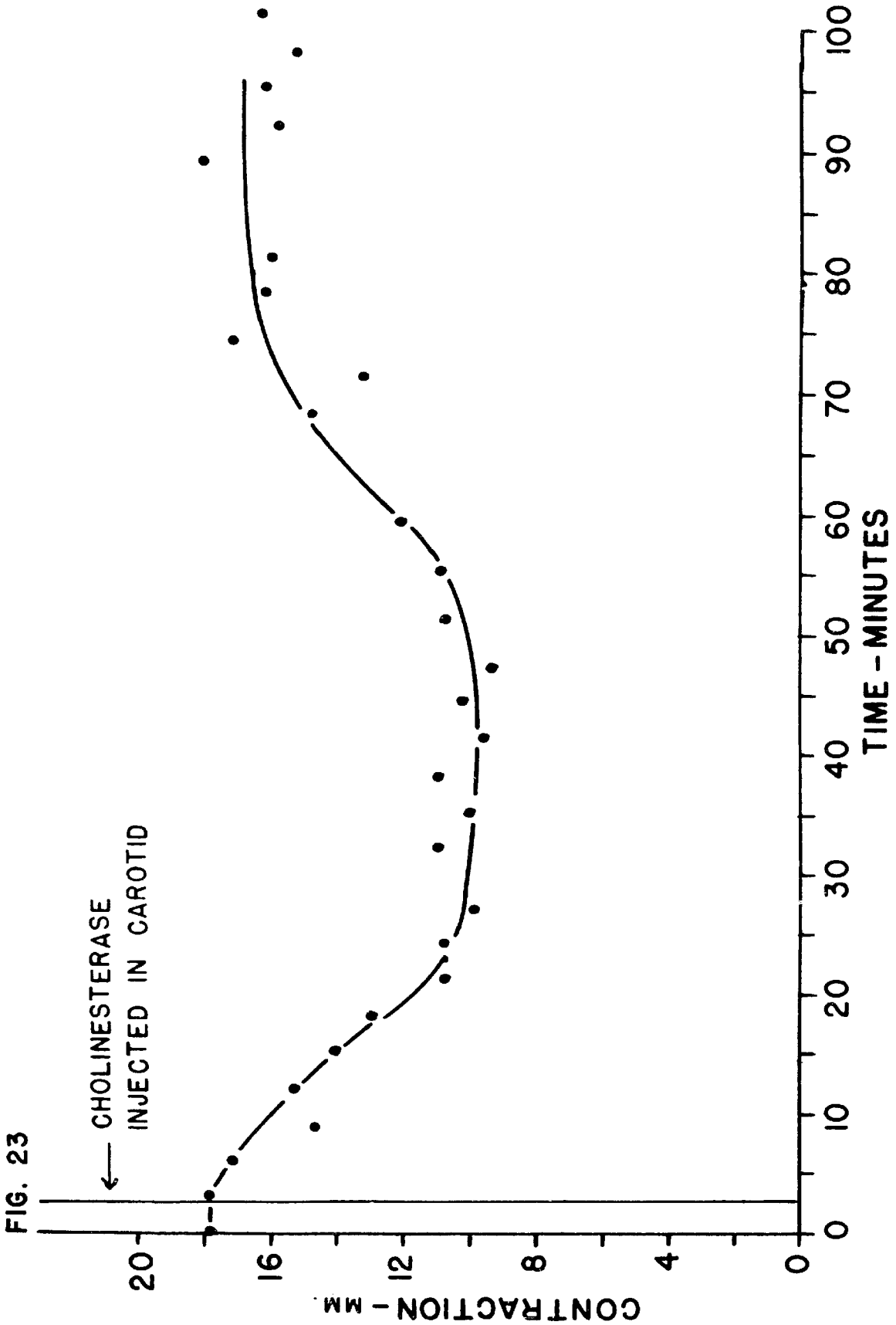

CHOLINESTERASE
INJECTED IN CAROTID
CONTRACTION - MM.
TIME - MINUTES
0
4
8
12
16
20
0
10
20
30
40
50
60
70
80
90
100

FIG. 23

indicate a high concentration of cholinesterase in the ganglion. One of the problems posed by past work is the effect of increasing the concentration of cholinesterase in the ganglion.

As the strength of stimulation to the preganglionic nerve was increased from threshhold to maximal, the contractions of the nictitating membrane increased until the maximum was reached. The number of the preganglionic nerve fibers stimulated would increase with the strength of stimulus until all of the fibers concerned with the nictitating membrane were being stimulated.

Varying the frequency of stimulation would cause the stimulated nerve fibers to fire according to the applied frequency, provided the frequency does not infringe on the refractory period. A theoretical frequency of approximately 500 shocks per second could be applied before conflicting with the refractory period of 2 milliseconds (Brown, 1934). The maximum frequency used in these experiments was 50 per second. Thus, unless the refractory period was changing by a factor of 10, the stimulated nerve was firing synchronously with the applied frequency.

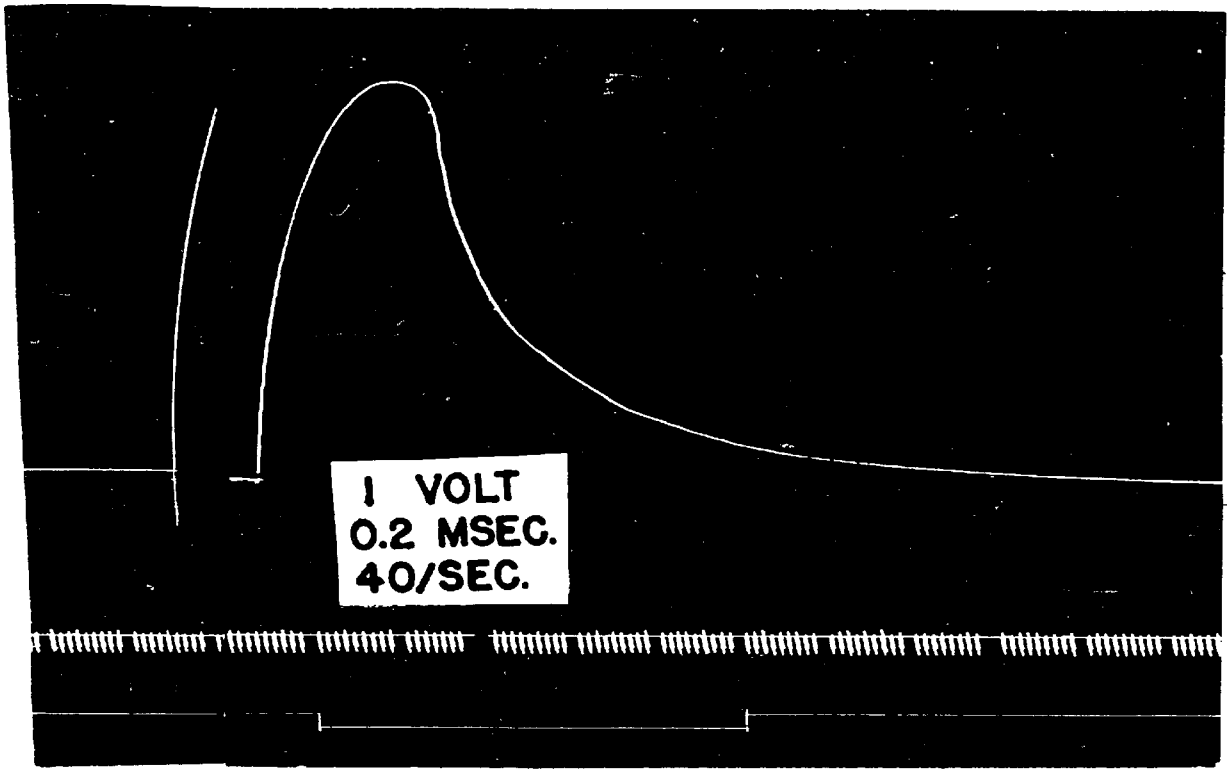
The frequency of stimulation applied to the preganglionic nerve depended on the type of experiment being conducted. When cholinesterase was injected into the circulation, only a single frequency was studied in most experiments since the time of action of the cholinesterase was obviously limited to the time of perfusion through the ganglion. When cholinesterase was applied topically, several different frequencies were studied with the same application of cholinesterase.

If frequencies higher than approximately 20 shocks per second were applied to the preganglionic nerve, the nictitating membrane responded with a decreased maximal contraction which subsequently declined even though the stimulation continued. Brown and Feldberg

(1936, b,c), stimulating the preganglionic nerve of an eserinized preparation for a prolonged period, found that acetylcholine is liberated in depressing amounts in the early part of the stimulation, thus producing a relaxation of the nictitating membrane. This data raised the question whether depressing amounts of acetylcholine could be the cause of the decreased maximum and subsequent decline when high frequencies of stimulation were applied. If such were the case, then application of cholinesterase, destroying the acetylcholine more rapidly, would tend to increase the contraction and decrease the amount of subsequent decrease because part of the depressing quantity of acetylcholine would have been eliminated. However, the results were contrary to this. The amount of contraction decreased (figures 17-22) and the subsequent decrease appeared to have no predictable correlation with the height of contraction (effect of cholinesterase). Brown and Feldberg were unable to obtain depressing quantities of acetylcholine if the preparation was not eserinized. Eserine was not administered in the present investigation. Therefore, it appears that the decreased maximum and the subsequent decline with higher frequencies is not due to the liberation of depressing amounts of acetylcholine.

Brown and Feldberg (1936,c) also found that the amount of acetylcholine liberated by the ganglion, using a frequency of 17 shocks per second or less, decreased exponentially. This frequency is below that usually required to produce a decrease after the initial maximal response of the nictitating membrane. Higher frequencies should cause the acetylcholine to disappear more rapidly. When the logarithm of the height of a contraction of the nictitating membrane during the declining phase is plotted against time (figure 24), a straight line results. Comparing this with the logarithmic decrease of acetylcholine of Brown and Feldberg suggests

Figure 24. A. A single contraction of the nictitating membrane due to a high frequency stimulation showing the declining phase.
B. Logarithm of contraction height plotted against time for the declining phase.


1 VOLT
0.2 MSEC.
40/SEC.

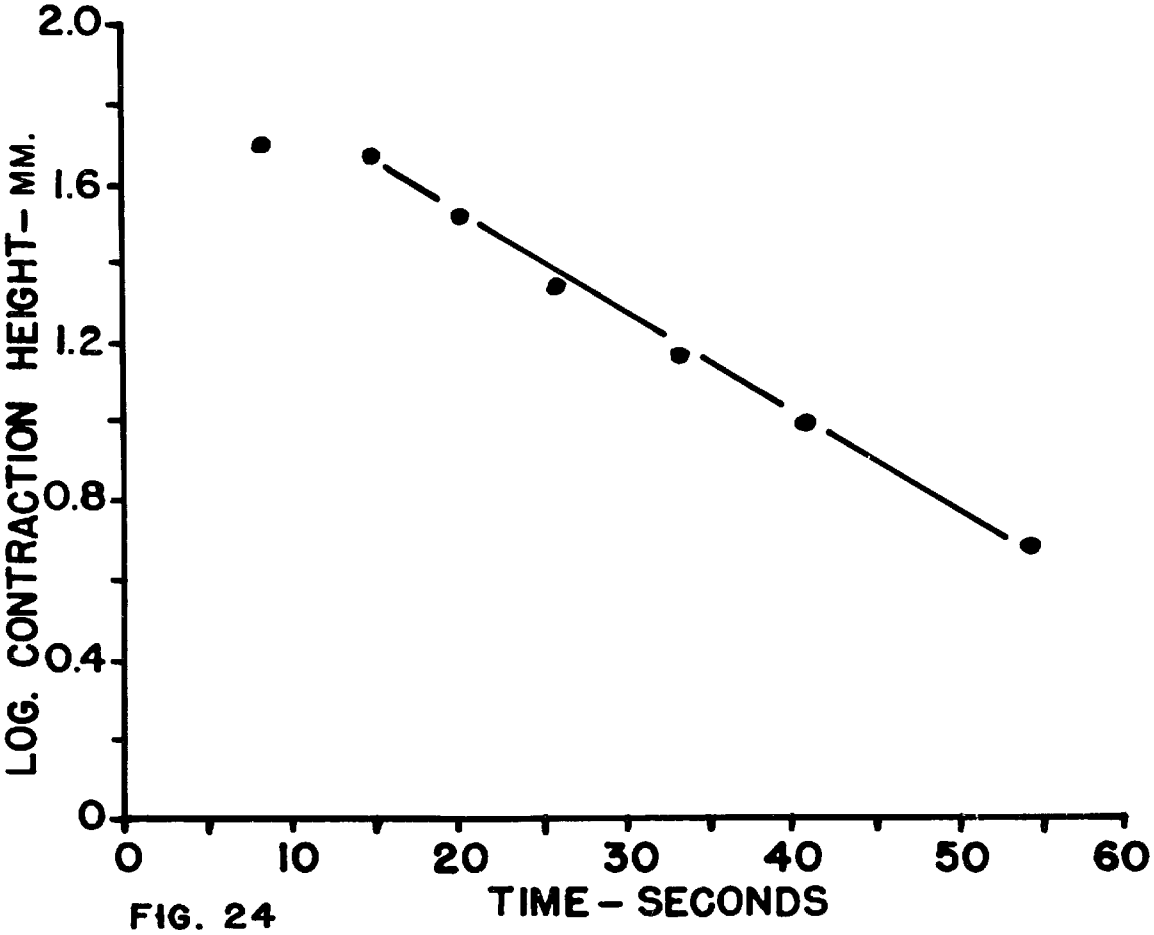

LOG. CONTRACTION HEIGHT – MM.
2.0
1.6
1.2
0.8
0.4
0
0
10
20
30
40
50
60
TIME – SECONDS

FIG. 24

that the decrease of available acetylcholine is the cause of the declining phase that occurs with high frequency stimulation. High frequencies would cause a faster decrease due to the increased number of stimuli. Thus, the decreased maximal height of contraction at high frequencies is an indication that the acetylcholine is being liberated and destroyed at a faster rate than it is being resynthesized.

Synthesis of acetylcholine by the ganglion was shown by Brown and Feldberg (1936,a), when they obtained more acetylcholine from the ganglion by preganglionic stimulation than could be extracted from the non-stimulated ganglion. Since the ability to synthesize acetylcholine is lost when the preganglionic fibers are allowed to degenerate, Feldberg (1943) reasoned that the synthesis was a property of the preganglionic fibers.

Cholinesterase, either injected or applied topically to the superior cervical ganglion, resulted in a decreased height of contraction of the nictitating membrane when the preganglionic sympathetic was stimulated. The effect from topical application was usually more pronounced than when the cholinesterase was injected into the circulatory system. However, the time required for the cholinesterase effect to occur was less when the injection method was used. Since the cholinesterase molecule is very large, diffusion from the blood vessels will only occur if there is a large diffusion gradient. That was the purpose of concentrating the entire injection into only the superior cervical ganglion. Diffusion into the ganglion by topical application is also understandably slow, considering the type of tissue and the high molecular weight of the cholinesterase.

In both methods of application, the greater decrease of height of contraction occurred at higher frequencies of stimulation. With

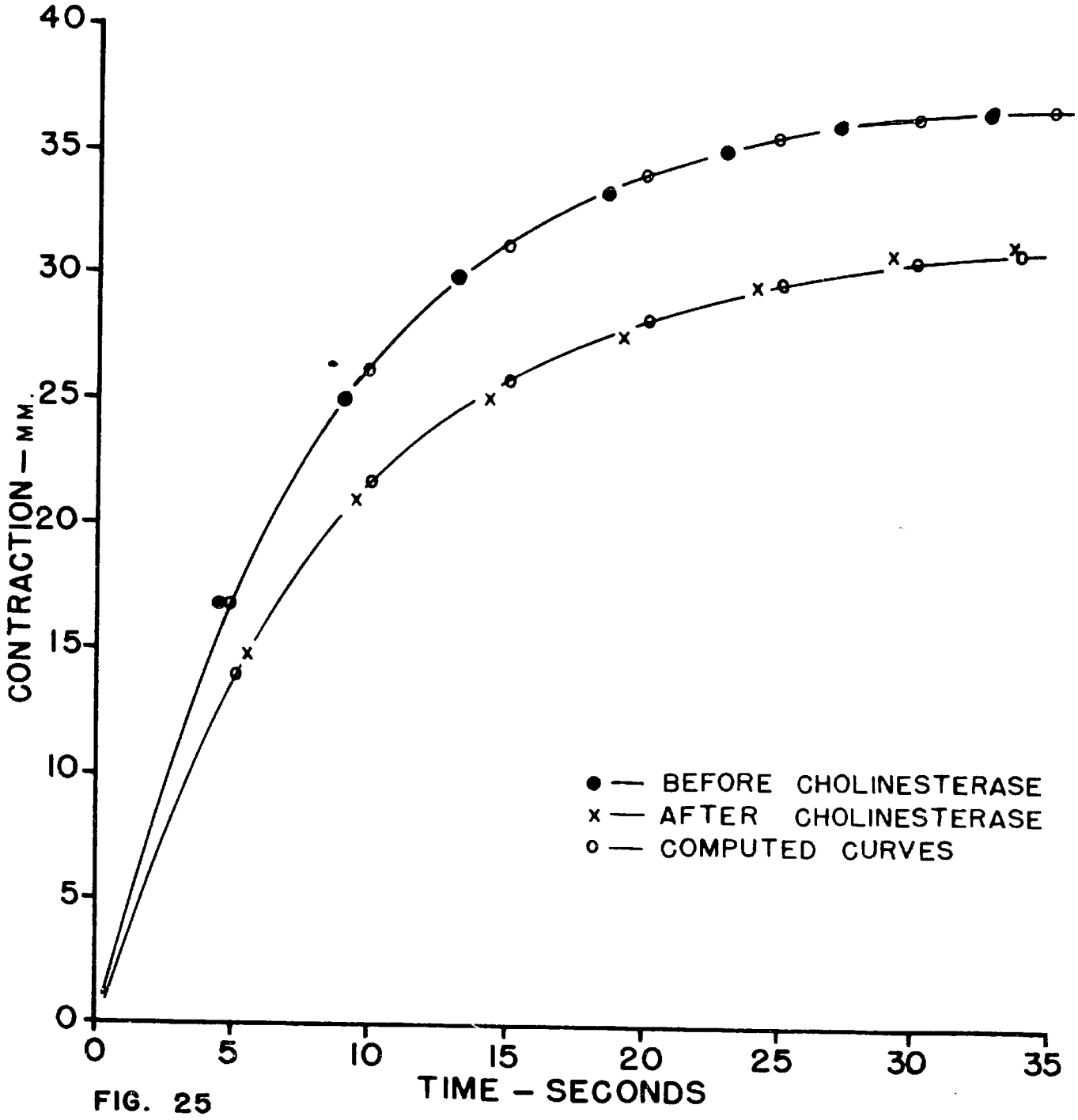
the injection method, comparison of the various frequencies of stimulation is difficult because they are done on different preparations. Two sets of data are presented, one at low frequency and one at a high frequency (figures 21 and 22). The maximal cholinesterase effect at the low frequency was 16.2 percent and at the high frequency was 43.8 percent. Also, the effect at the high frequency persisted for a longer time (about 50 minutes) than did the effect at the low frequency (about 20 minutes). Experiments of Brown and Feldberg (1936,c) indicated that the stimulation of the preganglionic nerve to the superior cervical ganglion caused a vasoconstriction of the blood vessels of the non-eserinized ganglion. Eserinizing the preparation sometimes caused vasodilation. Since the preparation used here was not eserinized, then preganglionic stimulation probably caused vasoconstriction. This would delay the passage of the cholinesterase through the ganglion. The cholinesterase would also enhance the vasoconstriction by destroying the acetylcholine, a vasodilating substance. Thus, at high frequencies, it is possible that greater vasoconstriction occurred due to the larger number of stimuli, allowing the cholinesterase to react longer. This is speculation that would require a measurement of the vasomotor effect for conformation. A further analysis of the problem of the greater effect at higher frequencies can more easily be dealt with in the cases in which the cholinesterase was applied topically.

When cholinesterase was applied topically, several different frequencies were studied with each application. The effect was progressive, the maximum occurring in 40-60 minutes. The greatest changes occurred at the higher frequencies, conforming with the observations of various frequencies on different preparations when cholinesterase was injected into the circulatory system.

After the contraction of the nictitating membrane attained a plateau during low frequency stimulation, the amount of acetylcholine being released with each individual stimulus should equal the amount being hydrolyzed between each stimulus. Curves were computed before and after cholinesterase in the same manner as outlined for the progressive inhibition of the turtle auricle with multiple stimulation to the vagus nerve (figure 25). These curves fit the experimental data very closely. Therefore, the height of the contraction at a low frequency stimulation is an indication of the level of acetylcholine in the ganglion. Cholinesterase applied to the preparation will cause the acetylcholine liberated with each stimulus at a low frequency to be destroyed faster. This will decrease the amount of acetylcholine present when the equilibrium is reached between the rate of destruction and the liberation of acetylcholine, just as did the inhibition of the turtle auricle when cholinesterase was applied.

At high frequencies, the contraction of the nictitating membrane should be the resultant of three actions, 1) the rate of hydrolysis of acetylcholine, 2) the rate at which the acetylcholine is liberated, and 3) the rate of synthesis of acetylcholine. At low frequencies, the liberation and destruction of acetylcholine does not occur as rapidly as the synthesis. Therefore, the contraction of the nicititating membrane will be correlated with the frequency of stimulation since there is always sufficient acetylcholine available. At high frequencies, the liberation and destruction of the acetylcholine is more rapid than the synthesis; therefore, the contraction of the nictitating membrane will be correlated with the frequency of stimulation until the supply of acetylcholine has begun to diminish. Then the contraction will only occur as the acetylcholine is being formed. If cholinesterase is added to such a

Figure 25. Computed curves versus the experimental data obtained from the experiment shown in figure 21. The contractions used were the control A before the cholinesterase was injected and the contraction when the maximal effect of cholinesterase occurred.


40
35
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25
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15
10
5
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CONTRACTION — MM.
0
5
10
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20
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TIME — SECONDS
● — BEFORE CHOLINESTERASE
x — AFTER CHOLINESTERASE
o — COMPUTED CURVES

FIG. 25

system, the destruction of the acetylcholine of each separate stimulus is increased. This means that the time, when the liberation and destruction of the acetylcholine becomes more rapid than the resynthesis, occurs sooner. Thus, there will be two effects tending to decrease the height of contraction from a high frequency stimulation, an increased rate of destruction from the cholinesterase and a decreased amount of acetylcholine available for each stimulus. Low frequency stimulation would involve only one effect, the increased rate of hydrolysis due to cholinesterase. Therefore, there will be a greater decrease when cholinesterase is applied at a high frequency than at a low frequency.

Summary

The contraction of the nictitating membrane of the cat was used as a criterion of the superior cervical ganglion activity when the preganglionic nerve was stimulated. When the frequency of stimulation was increased from approximately 2-20 shocks per second, the height of contraction increased until it reached a maximum. However, any further increase in the frequency of stimulation would cause the maximal height of contraction to decrease and following this, the height of contraction will decrease even though the stimulus was continued. The logarithmic relation between the height of contraction during this declining phase and time resembles the work of Brown and Feldberg in which they demonstrated that prolonged stimulation at a low frequency to the preganglionic nerve caused a logarithmic decrease in the amount of acetylcholine released with each stimulus. It appears that the resynthesis of acetylcholine plays an integral part in maintaining the necessary amount of acetylcholine available for liberation by nerve stimulation. The contraction of the nictitating membrane is dependent on the rate of liberation, the rate of hydrolysis, and the rate of

resynthesis of acetylcholine. If the liberation and the hydrolysis is not as rapid as the resynthesis, then there will always be sufficient acetylcholine. But if the resynthesis is not rapid enough to maintain the supply of acetylcholine at a given rate of hydrolysis and liberation, then the characteristic decrease of the maximal contraction and the declining phase will occur.

The effect of the cholinesterase, either topically or arterially, was to decrease the amount of contraction of the nictitating membrane. The higher the frequency of stimulation the greater was the effect of the cholinesterase. This appeared due to the result of two effects, an increased rate of hydrolysis and the decreased amount of available acetylcholine for liberation.

TEMPORAL SUMMATION

This investigation was instituted for two purposes. The first was to study general results of administering a cholinesterase preparation (Cutter Laboratory) to three specific cholinergic systems. This has been done and reported in the preceding sections.

The second purpose of the investigation was to supply, if possible, collaborative data for the support of the neurohumoral mechanism of temporal summation as proposed by Gesell's theory of neurocellular generation.

According to Gesell, there are five functions involved in the transmission of an impulse. The dendrites and the cell body function as a source of self-excitatory current. The axon hillock generates nerve impulses in response to the neurocellular current flowing through the neuromembrane. The axon conducts the generated impulse to the next neuron or to the end organ. The impulse of the axon is converted into non-propogated depolarizations at the end boutons. And finally, the nerve cell may be inhibited by synaptic depolarization at the axonic end of the cell. (Gesell, Brassfield, and Lillie, 1945).

The liberation of electrogenic acetylcholine by the end boutons at the dendrites or the dendritic end of the cell body serves the purpose of increasing the neurocellular current. When the current exceeds the threshhold of the axon hillock, an impulse is propogated down the axon. The monomolecular hydrolysis of acetylcholine in the area of release must be rapid in order to return the neurocellular current to normal. If successive units of acetylcholine are liberated by a repetitive stimulus, the acetylcholine will summate until a concentration is reached where monomolecular hydrolysis between each stimulus equals the liberation. This has been demonstrated by Beznak and Farkas (1937) for the temporal summation of submaxillary

secretion from chorda tympani stimulation, by Gesell and Frey (1950) for temporal summation of hyperpnea from carotid nerve stimulation of the respiratory centers in the central nervous system, and by Peters and Gesell (1952) for temporal summation of cardiac inhibition due to vagal stimulation.

According to this concept of temporal summation, cholinergic systems are regulated by the rate of liberation of acetylcholine (frequency of stimulation), rate of hydrolysis of acetylcholine, and possibly by the rate of synthesis of acetylcholine. Various means have been used to determine if temporal summation is produced by the accumulation of acetylcholine. Beznak and Farkas favorably compared different frequencies of stimulation of the chorda tympani with injections of acetylcholine. Gesell and Frey varied the frequency of stimulation and applied several anticholinesterases. Peters and Gesell used various frequencies of stimulation. Each of these procedures conformed with the predicted result if temporal summation is due to the accumulation of acetylcholine.

The present investigation tested the hypothesis by applying a cholinesterase to increase the rate of hydrolysis of the liberated acetylcholine. In each of the three systems studied, stimuli to the nerves caused less activity of the end organ when cholinesterase was administered. Increasing the rate of hydrolysis will hydrolyze more acetylcholine between successive stimuli; thus, the acetylcholine level will be less at the equilibrium point when the amount hydrolyzed equal the amount liberated. In other words, the temporal summation will be less.

The vago-cardiac system and the chorda tympani-submaxillary gland system are peripheral neuro-effector mechanisms. Since they have parasympathetic nerve supplies, the cholinesterase will have an opportunity to act in two places, at the neuro-effector junction

and at the postganglionic cell body. Increasing the rate of hydrolysis in either of these places should cause a decrease in the amount of temporal summation displayed by the end organ. Therefore, the final recorded cholinesterase effect is probably the sum of the decreased temporal summation at the postganglionic cell body and at the neuro-effector junction.

When the vagus nerve is stimulated with a repetitive stimulus, the inotropic inhibition of the auricle gradually increases until it reaches a maximum. The shape of the curve of this phenomenon conforms with the postulated curve based on the temporal accumulation of acetylcholine which is being hydrolyzed at the same time (figure 11). When cholinesterase was applied to the system, the postulated curve had to assume an increased rate of hydrolysis to fit the experimental data. Therefore, it appears that the gradual increase of inhibition of the turtle auricle is due to temporal summation of quantities of acetylcholine, the summation being less when the hydrolysis is greater.

The superior cervical ganglion contains a cholinergic system within itself, the cholinergic preganglionic nerve fibers affecting the postganglionic cell body to produce adrenergic postaxonic discharges to the end organ. With this mechanism, the cholinesterase will affect only the point of the liberation of acetylcholine, the preganglionic synapse with the cell body. The effector organ selected (nictitating membrane) has only adrenergic postganglionic innervation, thus eliminating any possible cholinesterase effect.

At low frequency stimulation (approximately 20 per second or less) of the preganglionic sympathetic nerve to the superior cervical ganglion, the temporal summation of the contraction of the nictitating membrane produced the same type of curve (figure 25) as the inhibition of the auricle from repetitive stimulation of the vagus.

High frequency stimulation of the preganglionic fibers to the superior cervical ganglion caused a contraction that subsequently decreased after reaching a maximum. This phenomena, discussed under a previous heading, appeared related to the synthesis of acetylcholine as well as the liberation and destruction. The relation of temporal summation to the reaction of the nictitating membrane at high frequency necessitates including the possibility that the amount of acetylcholine liberated with each stimulus to the superior cervical ganglion is decreasing. Such a decrease means that the amount of acetylcholine hydrolyzed between successive stimuli will also be less when the point of equilibrium is reached. Therefore, the effect of decreasing the amount of acetylcholine with each stimulus is to decrease the temporal summation. It was seen from the experimental results that high frequencies did decrease the maximal height of contraction of the nictitating membrane and caused a subsequent decline. This decline can also be due to the decrease of acetylcholine liberated with each stimulus. Two theoretical curves have been constructed in the same manner as previously. One was of a normal contraction having a constant amount of acetylcholine liberated at each theoretical stimulus and the other was based on a declining amount of acetylcholine liberated with each stimulus until the amount hydrolyzed is equal to the amount resynthesized (figure 26). These curves show the decreased maximal height of the theoretical contraction and the subsequent decline with decreasing amounts of acetylcholine. Therefore, the temporal summation of the nictitating membrane at high frequency stimulation of the preganglionic sympathetic nerve is probably due to the decrease of acetylcholine liberated with each stimulus.

Beznak and Farkas (1937) found that temporal summation of salivary secretion of the submaxillary gland by repetitive chorda

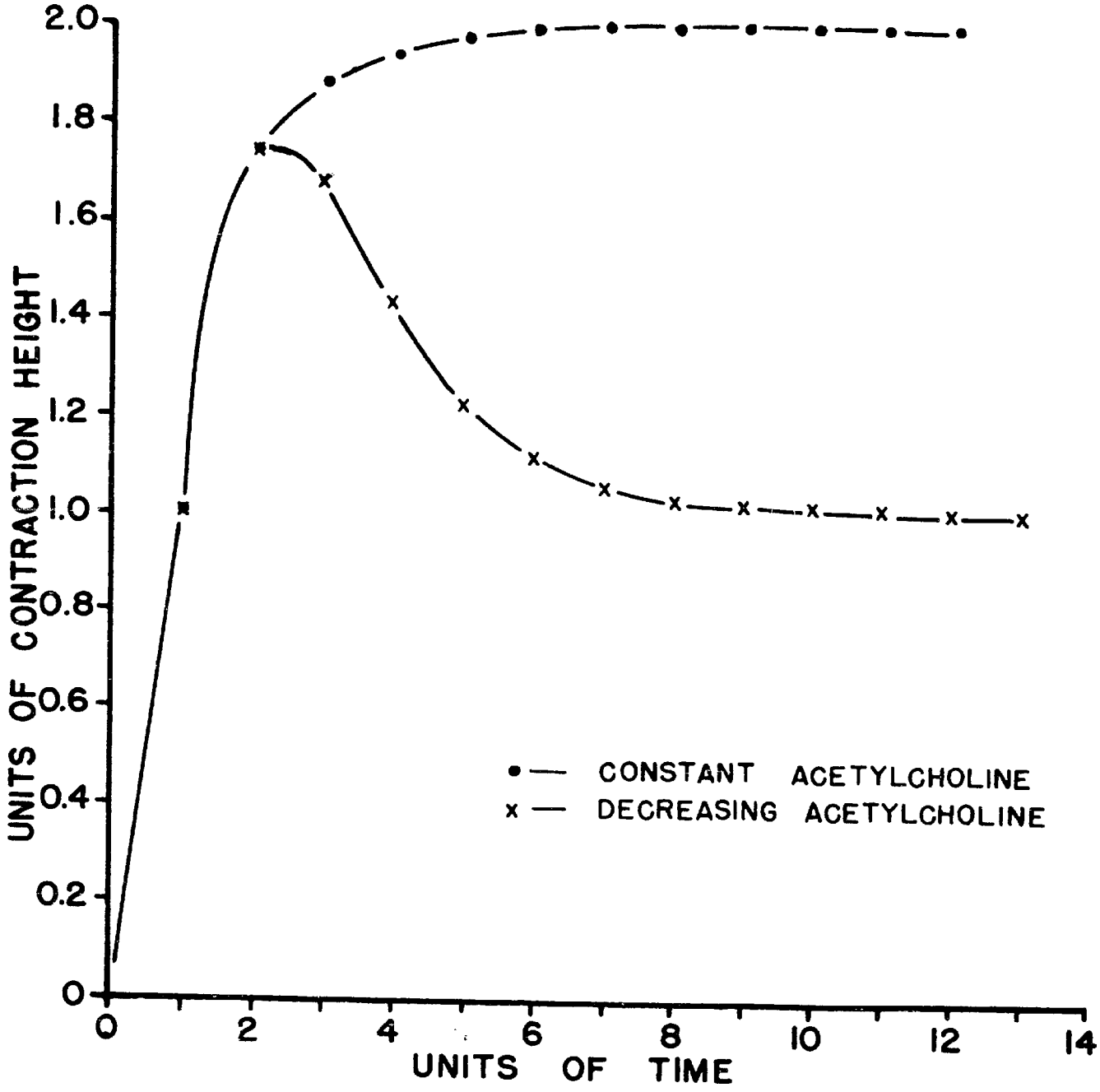

Figure 26. Theoretical curves showing the shape of the contraction of the nictitating membrane with low frequency stimulation (dots) and high frequency stimulation (crosses) to the preganglionic sympathetic nerve. Low frequency stimulation assumes a constant amount of acetylcholine liberated with each stimulus. High frequency stimulation assumes a decreasing amount of acetylcholine liberated with each stimulus.

tympani stimulation was consistent with the view that the quantity of acetylcholine liberated with each stimulus was adding to the existing level of acetylcholine while constant hydrolysis was occurring. This evidence of Beznak and Farkas plus the present experiments showing less increase in salivation and blood flow from chorda tympani stimulation when cholinesterase was applied, indicates again that temporal summation is a result of the accumulation of acetylcholine.

APPENDIX

METHOD

Vago-cardiac System

The head of a snapping turtle (Chelydra serpentina) was crushed and the spinal cord pithed as follows: Starting at the head a heavy wire was inserted in the spinal canal as far as the connection of the cervical vertebrae with the more rigid vertebrae of the carapace. The spinal column was severed with a large pair of bone scissors in the region of the connection of the cervical and lumbar vertebrae. Then the wire was inserted at this point and pushed as far posteriorly as possible. Though the spinal cord in the tail was not destroyed, the method did a complete job of pithing in the vital region which controls the pectoral muscles.

The plastron was removed by cutting along the sutures on each extreme edge of the ventral side of the turtle and by severing its connections with the skin and underlying muscle. Both forelegs were removed. In the intact animal experiments this eliminated interfering muscle twitches due to drying and artificial stimulation. With the excised heart preparation, it is easier to dissect the animal when the legs are removed. If the heart was used intact, the visceral peritoneum was usually cut to eliminate its interfering twitches and tonus changes resulting from drying and deterioration.

The first experiments were done on the right auricle intact in the animal. The pericardium was laid open to form a pouch to contain the ambient fluid. The right vagus nerve was exposed in the neck and placed in a moist chamber with platinum electrodes. The right auricle was connected to a muscle lever scribing on a kymograph. This preparation proved unsatisfactory because of an inadequate means of changing the solutions, interfering action of other tissues, and difficulty of preparation.

More successful experiments were conducted on an excised heart preparation. This was made as follows: The plastron was removed and then the forelegs. A portion of the right vagus nerve about 3 centimeters long was isolated high in the neck and tied with a silk ligature. Another ligature was tied centrally to this, then the whole carotid sheath was separated from the underlying tissue as far posteriorly as possible. The left carotid sheath with the enclosed left vagus nerve was tied about the mid-point on the neck and separated from the underlying tissue as was the right vagus. The muscular sheet of the anterior end of the visceral cavity was incised to expose the lungs. A small portion (0.5 cm length) of lung tissue where the trachea enters the lung was removed with the preparation. Then, by lifting the vagi together, it was possible to sever the remaining part of the pericardial sac with the heart enclosed. A small portion (0.5 cubic centimeter) of the right lobe of the liver was left intact to allow absolute identification of the right auricle when the preparation was completed. The preparation was placed on a glass plate and the pericardial sac removed as much as possible. In the majority of such preparations the ventricle was removed by cutting along the easily identified auriculoventricular border. Also the left auricle was usually incised to prevent the action of filling from affecting the base line on the kymograph as much as possible. The right auricle was anchored to a glass tube formed to suspend the preparation in a 150 cc. glass receptacle containing 100 cc. of perfusing solution. A small pin was inserted in the right auricle and a thread from the pin was attached to the muscle lever. The distance from the lever to the glass supporting rod was about 20 centimeters. The magnification of the lever was usually between 10:1 and 10:1.5. The dissected right vagus nerve was separated from the carotid sheath and clipped just anterior to

the attached ligature. The freed nerve was then placed in a salt bridge electrode for stimulation. Time was recorded in seconds by a signal magnet.

The original nerve moist chamber with its enclosed platinum electrodes proved a source of error. Several stimulations of sufficient strength to give an inhibition of the auricle resulted in a "burned" nerve. The nerve became brown in the area of the small platinum electrodes and would no longer conduct an impulse. It was felt that the current density of the small platinum electrodes (less than 1.0 mm. diameter) was so great that with successive stimulations, the nerve was destroyed. Thus the vagal stimulation was not constant. A reversible silver-silver chloride electrode was devised (figure 27 and 28).

A piece of lucite plastic 7.5 cm. x 2.5 cm. x 1.2 cm. was used for the construction. Three holes (0.7 cm. diameter) were drilled, starting 0.2 cm. from one end of the block, on the 1.2 cm. side. The holes were 0.2 cm. apart and 2.0 cm. deep. Then a groove, 0.2 cm. wide and 0.7 cm. deep was machined through the center of the holes for a distance of 3.5 cm. along the 1.2 cm. side of the block. The nerve was placed in this groove so that it stretched across the three holes. A notch, 0.3 cm. deep and 3.0 cm. long was cut from the top side (1.2 cm. side) of the plastic piece to allow a cover to be fitted. The cover was made the same shape as the notch (0.3 cm. x 1.2 cm. x 3.0 cm.) and the small portion of the groove that extended beyond the notch for 0.5 cm. This small projection served to hold the cover in place. On the cover in position to fit down into the first and third holes were flanges that projected 0.6 cm. into the holes. The purpose of these projections was to force the nerve down into the fluid contained in the hole. Two small coils of silver wire (18 gauge) were fitted into the bottom of

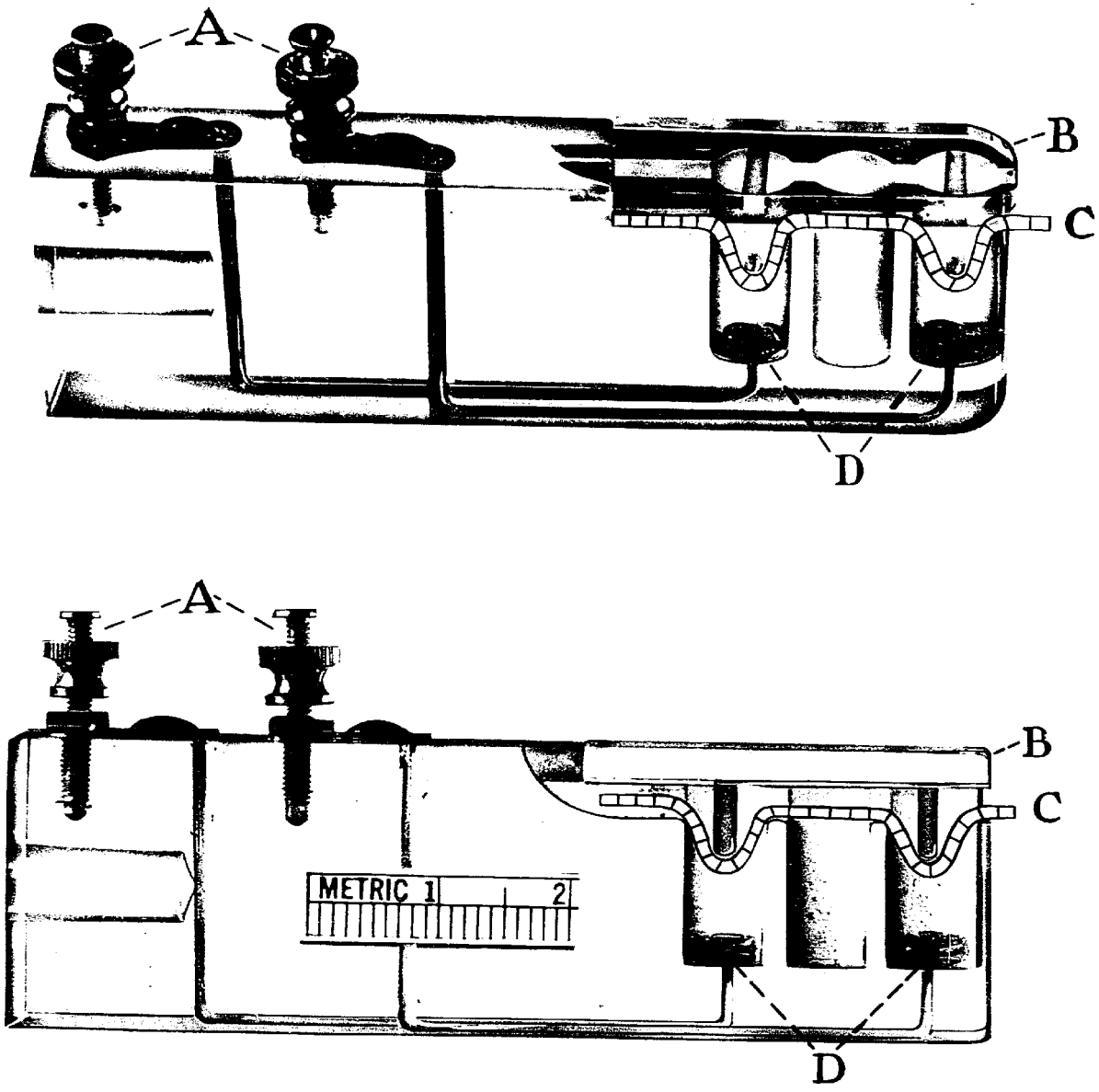

Figure 27. Two views of the reversible fluid bridge electrode used for stimulation of the vagus nerve.
A. Connecting posts for the stimulator.
B. Removable lid.
C. Position of the nerve.
D. Silver electrodes, chloridized.

Figure 28. Detailed plan of the reversible electrode used for stimulation of the vagus nerve.

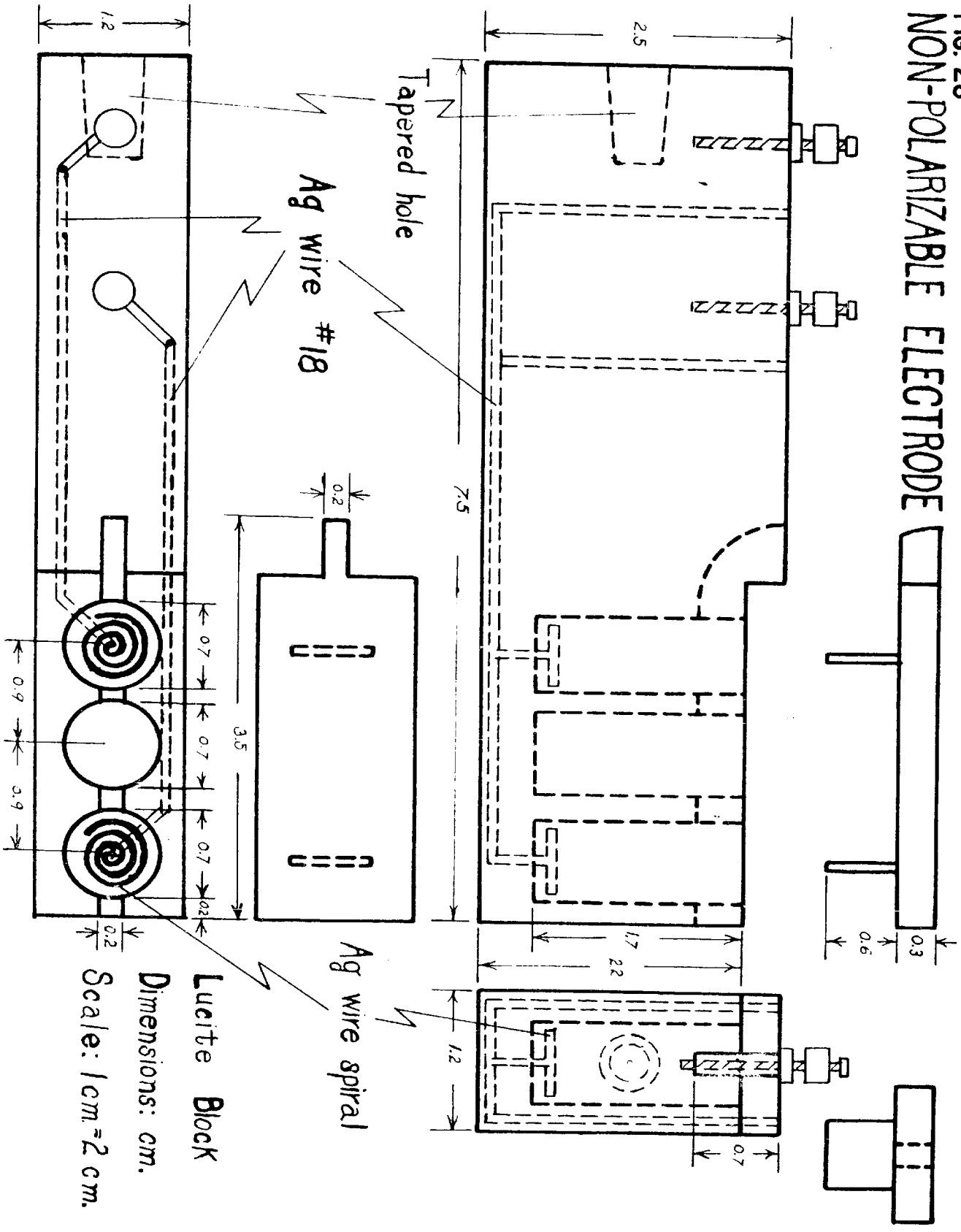

FIG. 28
NON-POLARIZABLE ELECTRODE

the first and third holes with the wire coming out a small hole in the floor of the tube, then being bent back to lie in a shallow groove on the bottom of the plastic block. This wire was then put through fine holes to the top of the block to allow easy connection with the stimulator. The grooves containing the wire were filled with plastic to prevent shorting and also to make the large holes water-tight. A tapered hole was placed on the rear of the block for a supporting rod.

The first and third holes were filled with inorganic salt solution and the silver electrodes were chloridized. The solution was then replaced and the electrodes were ready for use. The nerve was placed in the groove so that it extended beyond the third hole. Then the cover was placed on top, the flange pushing the nerve well down into the solution and the small extended portion serving to hold the cover and the nerve in place.

A square wave stimulator having frequency, duration and strength controls was attached to the electrode. This method of stimulation was very satisfactory as a "burned" nerve was never again encountered.

An attempt was made to use plasma, pericardial fluid, Ringer's solution, Howell's solution, and Clark's solution as the ambient solution. The only one that maintained a steady heartbeat was the pericardial fluid. But there was not enough to run controls before, during and after cholinesterase. Therefore, a new physiological salt solution was prepared on the basis of Homer Smith's (1929) analysis of Chelydra serpentina body fluids, namely, pericardial fluid and plasma. A gram of glucose was also added to each liter of solution. If the entire amount of calcium as determined by H. Smith was added to the solution, a precipitate occurred which was probably calcium carbonate. It was found by

trial and error that approximately one-half of the computed $CaCl_2$-$2H_2O$ would not give a precipitate. The other calcium may have been held by a protein complex in the animal fluid tested. Pericardial fluid was tested qualitatively for protein and found to contain some. The solutions were prepared as follows:

TABLE 4

Based on:	Grams per Liter					
	NaCl	NaH_2PO_4-H_2O	$NaHCO_3$	KCl	$CaCl_2$-$2H_2O$	Glucose
Plasma	3.74	0.24	4.4	0.23	0.40	1.0
Pericardial fluid	0.43	0.21	11.0	0.23	0.20	1.0

Chorda Tympani-Submaxillary Gland System

Medium sized dogs were anesthetized with urethane-morphine. The skin was incised from the base of the neck to the symphysis of the mandibles. Then a systematic investigation of the carotid artery and its branches on one side was made to determine the course of the arterial blood flow to the submaxillary gland. The vessels supplying this gland were, in general, from four sources and of these, at least two were always present.

The posterior thyroid artery, often arising from the common carotid artery, sometimes supplied a branch that entered the posterior part of the submaxillary gland. It seemed to be present mainly when there was a vein arising from the same area.

A second branch sometimes arose from the anterior thyroid artery and entered the gland on the medial surface, near or in the hilus of the gland.

One branch which was always present arose from the external carotid soon after the point where the external maxillary divides from the external carotid. This vessel turned caudalward and

followed Wharton's duct and the chorda tympani nerve into the hilus of the gland.

A fourth vessel, which was also always present, was a branch of the external maxillary artery. This branch was usually very small and entered the dorsal edge of the gland.

In the experiments, all arteries to the gland were left intact. All other branches of the common carotid, external carotid, and external maxillary arteries were tied. Injections were made either into the cannulated lingual artery or by hypodermic needle (#27) into the common carotid artery. This procedure insured a concentrated supply of the injected drug to the gland.

The lingual nerve with the accompanying chorda tympani was isolated as far centrally as possible. Stimulating electrodes were attached to the peripheral cut end of the nerve. The position of the electrodes was not changed throughout the entire experiment.

To record salivary flow, Wharton's duct was cannulated. The cannula was attached to a small Erlenmeyer flask completely filled with saline solution and tightly stoppered. A second tube connected the Erlenmeyer flask to an electronic drop recorder.

All veins to the external jugular vein except those from the submaxillary gland were ligated. The external jugular vein was cannulated with a "T" cannula. One outlet was attached to a large bottle completely filled with tap water so that the inflowing blood was deposited near the bottom of the bottle. Then, as with the salivary flow, a tube was connected from the top of the bottle to a drop recorder. The other opening of the "T" cannula was attached to an elevated citrate bottle for flushing the entire system.

Blood pressure was recorded from the femoral artery by a mercuric manometer. A respiratory record was obtained with a Hutchinson spirometer. All of these records were recorded on a

kymograph.

The chorda tympani was stimulated with tetanic shocks for a series of control periods. Then cholinesterase was injected into the carotid artery. Following this the chorda tympani was again stimulated at intervals to show the effect of the cholinesterase. The effect was noted until recovery was observed.

Superior Cervical Ganglion

The third system selected for study was the superior cervical ganglion of the cat. The cats were anesthetized with pentobarbital sodium, 40 milligrams per kilogram weight, intraperitoneally.

The preparation of the ganglion was made in one of two ways to facilitate the administration of cholinesterase. If care was taken, a small pocket could be formed around the ganglion by blunt dissection. As much connective tissue as possible was removed from the ganglion without interfering with the blood supply. The ambient solution was placed in the depression to surround the ganglion. Removal was accomplished with a syringe or absorbent cotton, depending on whether or not the fluid was saved.

The second method was perfusion of the ganglion through the intact circulatory system. This was accomplished by a modification of Kibjakow's method (1933). Whereas Kibjakow completely isolated the circulation of the superior cervical ganglion, it was desired in the present work to maintain the blood supply and yet administer a concentration of cholinesterase to the ganglion. Therefore, the common carotid artery was not cannulated as did Kibjakow. All blood vessels leaving the common carotid artery up to the carotid sinus were ligated. A careful investigation of the arteries in the area of the carotid sinus usually revealed a branch going to the superior cervical ganglion. The application of a few drops of dilute acetylcholine to dilate the vessels to a size that could be observed often

aided in the location of this branch. The internal carotid and all other vessels except the one to the ganglion were ligated. The external carotid was then tied off above the lingual artery. The lingual artery was also ligated about 0.5 centimeter from the external carotid. Then a bulldog clamp was placed on the common carotid artery and a small hole cut in the lingual artery. A 10 centimeter piece of polyethylene tubing (#10) was threaded through this hole until the end lay on the central side of the carotid sinus. A ligature was then placed around the external carotid as close as possible to the carotid sinus. This served to hold the tubing in place and to channel all of the injected material into the carotid sinus and hence to the superior cervical ganglion. A second supporting ligature was also placed around the lingual artery. The bulldog clamp was removed as soon as possible to minimize anoxia. A needle (#27) was placed in the polyethylene tube and a sufficient amount of 0.9 percent saline injected to fill the tube. This was never more than 0.02 cc. Then a hemostat was placed on the tubing before removing the syringe to prevent the blood from flowing out the tube. Before the injections were made, a small quantity of blood was allowed to drip from the end of the tubing to prevent the action of any residual material and to assure a free passage.

When any solution was injected, a bulldog clamp was placed on the common carotid artery during the injection to keep the solution concentrated in the area of the carotid sinus, thus minimizing the dilution by blood. Allowing the bulldog clamp to remain on the artery only a few seconds did not cause a recordable effect from anoxia.

Small amounts of acetylcholine were injected to determine if all vessels except the ones to the superior cervical ganglion were occluded. If not, facial and cervical muscle twitches occurred as

well as the contraction of the nictitating membrane. Reinvestigation usually revealed a free artery that was subsequently ligated. At the end of the experiment, an amount of dye (usually fast green) equal to the amount of cholinesterase used was injected in the same manner as the cholinesterase to verify whether the ganglion was being adequately perfused.

All solutions were warmed to 37-39 degrees centigrade before applying to the ganglion, either topically or arterially.

The preganglionic fibers of the sympathetic to the superior cervical ganglion were isolated for approximately 3-4 centimeters and placed in a reversible fluid electrode, similar to the design of Collison (1933) (figure 29). The purpose and the principles of this electrode are essentially the same as those of the reversible electrode used for the stimulation of the vagus nerve of Chelydra serpentina.

Isotonic contractions of the nictitating membrane, recording on a kymograph, were used as an indication of the activity of the ganglion when the preganglionic fibers were stimulated. The nictitating membrane was selected because its postganglionic innervation is entirely adrenergic and thus not subject to the injected cholinesterase.

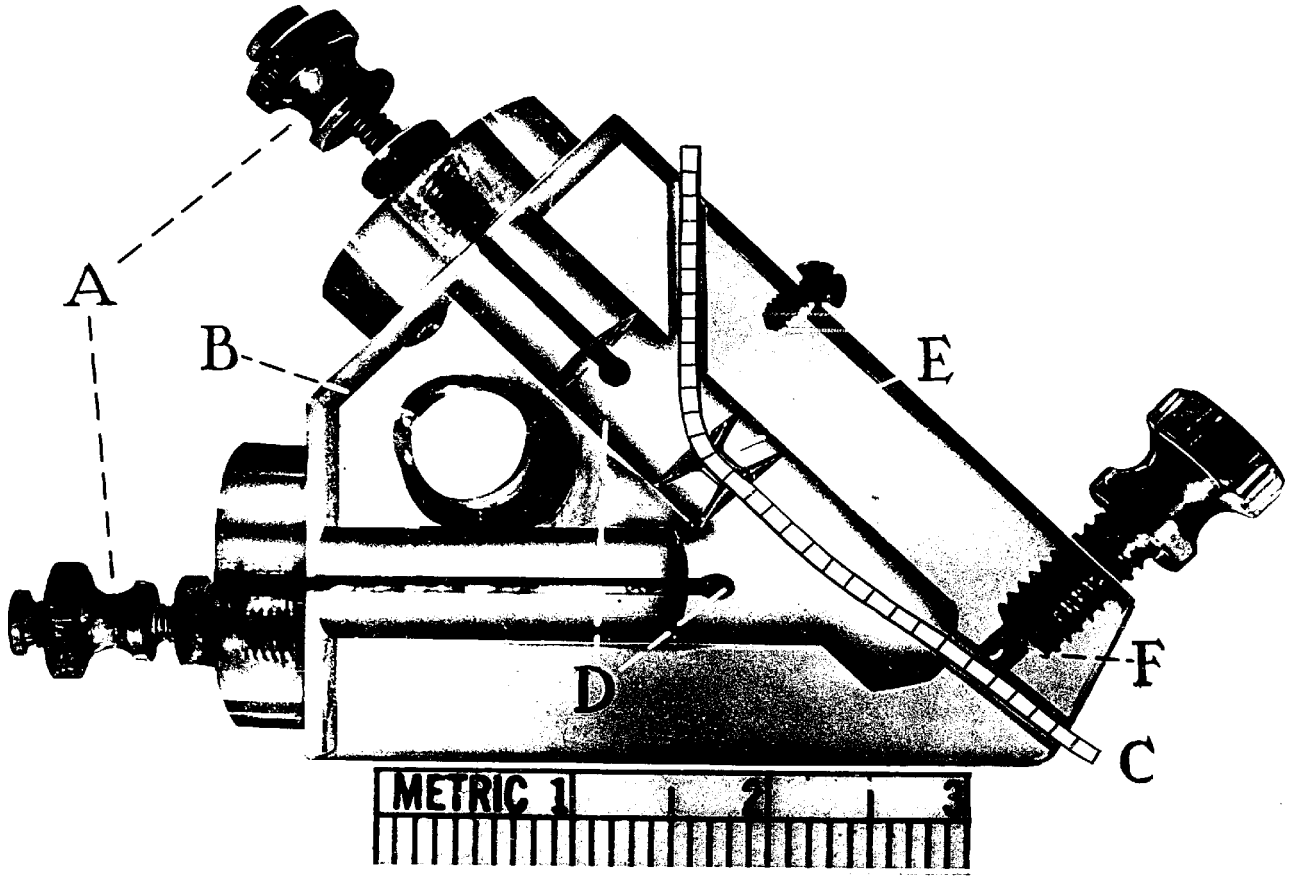

Figure 29. Photograph of the reversible fluid bridge electrode used for stimulating the cervical sympathetic.
A. Connecting posts for stimulator.
B. Tapered hole for a supporting rod.
C. Position of the nerve.
D. Silver electrodes, chloridized.
E. Spacer separating the electrodes.
F. Grease cup

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