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The Effects of Magnesium on the Recovery and Assay of Acetylcholine Liberated by Motor Nerve Stimulation

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THE EFFECTS OF MAGNESIUM ON THE RECOVERY AND ASSAY OF ACETYLCHOLINE LIBERATED BY MOTOR NERVE STIMULATION.

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INTRODUCTION

As early as 1904, Elliott suggested that the sympathetic nerve fibers might act by the liberation of adrenalin at the nerve endings. However, it was not until 1921 before Loewi's classical frog-heart experiments placed the existence of a chemical mediator beyond a reasonable doubt. Since then, extensive work has been done in an effort to apply the humoral theory of autonomic nerve-muscle transmission, to include also the peripheral motor nerve-stiated muscle transmission.

In 1934, experiments conducted on the cat's tongue by Dale, Feldberg, and Vogt, have shown quite conclusively that Acetylcholine is liberated at the endings of a stimulated motor nerve. They also found that curare, although abolishing the muscular response to nerve stimulation, did not abolish Acetylcholine liberation. The last mentioned research was definitely instrumental in shifting the balance of sentiment toward the chemical hypothesis.

Even at present, the problem of neuro-muscular transmission is being investigated by two opposing groups: 1) the Acetylcholine hypothesis, and 2) the electrical action current hypothesis, (Eccles 1935). Although, there is evidence favoring both these theories, no experiment has yet been devised that would definitely exclude either one or the other of these theories.

Work of Lubinska (1935), and more recently, that

Brosnan and Boyd (1936) on preparations "curarized with magnesium sulphate," showed that repetitive stimulation of a motor nerve temporarily decurarized the preparation, and in general behaved in a manner strenghtening the existing chemical hypothesis of muscle-nerve transmission.

The research herein described is entirely based upon the chemical theory of intermediary transmission, and was performed to gain further evidence for or against the presumptive findings of the above workers. Although we have worked primarily on the recovery and assay of Acetylcholine from magnesium "curarized" and eserinized preparations, we have deviated enough to warrant the following classification:

I. Assay for Acetylcholine from eserinized muscle-nerve preparations, "curarized" with curare.
   (a) Using the natural circulation.
   (b) Using the perfusion method.

2. Assay for Acetylcholine from eserinized and "curarized" preparations, using $MgSO_4$ as the blocking agent. (this group comprises the greatest bulk of our work).

3. Assay for Acetylcholine using non-eserinized, but magnesium "curarized" preparations, to which eserine was added within 15-30 seconds of stimulation of the motor nerve.

Acetylcholine, which was first prepared in 1867, has

the formula HO. N. $(CH_3)_3$. C_2H_4 $OCOCH_3$. It is an extremely hygroscopic substance, is rapidly hydrolyzed in alkaline or aqueous solutions, but is stable in acid or alcohol(Evans,1936). Physiologically, it has parasympathomimetic action.

METHODS AND MATERIAL

The detailed proceedure employed in the perfusion of the tongue, and in the assay for contained Acetylcholine in the perfusate was as follows:

Dogs were anesthetized with nembutal, and the lingual artery and vein on one side of the throat were cannulated. After injecting IO mg. of heparin into the cannulated lingual artery to prevent clotting, the tongue perfusion was started,using the perfusion pump. As rapidly as possible, the lingual artery and vein on the opposite side were ligated, and the carotid artery on the same side cannulated to allow bleeding of the animal. Rich anastamotic communications of the tongue made bleeding necessary to prevent contamination of the perfusion fluid with blood. These same anastamotic channels were responsible for the loss of an undetermined volume of perfusion fluid, which presumably was constant in each experiment.

The perfusion was continued until all traces of blood disappeared from the venous effluent. For this reason, anywhere from 3 to I5 minutes elapsed before a resting sample was taken for analysis. The rate of perfusion in the different experiments varied from 4 to 9 c.c. per minute, but was rather constant for each individual experiment.

Shielded electrodes were placed on the hypoglossal nerve of the perfused side, and the nerve stimulated with an induced current at a frequency of IOO per second. The muscle-

nerve preparation in all instances was completely "curarized" preliminary to the period of stimulation, to thus prevent all muscle response.

The perfusion fluid used, consisted of Locke's solution to which eserine (I-500,000) and magnesium sulphate or chloride, 80 to I00mgs. per cent had been added. The fluid was always preoxygenated, and warmed to 37.5°C.

The amount of magnesium added to the perfusion fluid determined the percentage concentrations of the other inorganic ions, so as to keep the fluid osmotically equivalent to normal Locke's solution. In a series of six experiments, a modified Locke's solution was used.

Sodium chloride----------0.87 per cent
Potassium chloride----------0.052 per cent
Calcium chloride----------0.028 per cent
Magnesium chloride----------0.I0 per cent

As soon as the perfusate is free of blood, a resting sample of I0 c. c. was collected, stabilized with trichloracetic acid, and placed on ice to prevent destruction of the active substance. Other I0 c.c. samples were collected during tetanization, and at varying intervals thereafter.

The assay for the Acetylcholine was usually carried out within 24 hours, and three proceedures were used in assaying the perfusates: the reactions of the dorsal body wall of the leech, the reactions of the frog rectus abdominis, and the

changes in cat's blood pressure. All these preparations are highly sensitive to Acetylcholine, and gave quantitative results which were in close agreement.

When using the rectus or leech muscles, the method of assay consisted of comparing the degrees of contracture produced by known quantities of Acetylcholine with contractures obtained with perfusates collected at various stages of the experiment.

The leech or rectus muscle was suspended in a muscle chamber containing I2 c. c. of Ringer's solution and was placed under a 4 gm. tension. A sensitive lever, arranged to magnify movements I2 times, was used to record movements of the muscle on a kymograph (Chang and Gaddum, I933).

After eserinizing the test preparation (0.2 mg. eserine salicylate applied for one-half hour) to increase its sensitivity for Acetylcholine, IO c.c. of the perfusion fluid properly diluted with distilled water was applied to the muscle, and allowed to remain in contact with the preparation for 2 to 3 minutes. Dilution of the perfusate with water was necessary to diminish the concentration of salts present in mammalian Locke's, to a concentration more suitable for survival and activity of the frog and leech muscles.

After removing the unknown solution, and allowing the preparation to relax, various graded doses of known Acetylcholine concentration were applied, so that a quantitative es-

timate could be made by comparing heights of contraction produced by the known and unknown solutions.

Two different methods of applying the perfusates to the test preparations were employed.

(a) Direct application of the IO c.c. doses of diluted perfusion fluid (Used only on the leech and recti preparations)

(b) Application of small volumes of concentrated perfusates. This method consisted of evaporating total volume of fluid to dryness (in vacuo), taking up with alcohol,again evaporating to dryness, and finally dissolving the residue with a small volume of saline, and testing. This method is especially useful when using the cats blood pressure preparation.

Since responses of both the rectus and leech muscles are depressed by the presence of magnesium ions, it was necessary to add to the standard Acetylcholine solution the same concentration of magnesium as the perfusate contained, to thus make possible satisfactory comparisons. Upon washing out the magnesium containing standard, recovery of the test preparation occurs, and thus permits repeated doses of the unknown solution to be assayed without any serious depreciation in the accuracy of the preparation.

The use of the leech and recti muscles is especially valuable in differentiating Acetylcholine effects from those elicited by potassium, which at present has also received serious consideration as being involved in some phase of the

neuro-muscular transmission (Cowan,I934).

Although, both Acetylcholine and potassium cause a contracture of the frog rectus muscle, only Acetylcholine is capable of eliciting a similar action on the leech muscle. The potassium actually causes a depression of the leech preparation (Chang and Gaddum, I933). Therefore, the concurrent use of both these preparations in assaying the perfusates, eliminates the danger of confusing Acetylcholine effects with those of potassium.

The other method used for assaying the Acetylcholine content of the perfusates, involved the measurment of the cat's blood pressure. A continuous record of the carotid blood pressure of a cat under nembutal anesthesia was made, and from 0.5-I.0 c.c. quantities of concentrated perfusion fluid, collected at various stages of the experiment,were injected intravenously. Presence of Acetylcholine elicited an immediate, temporary drop in the blood pressure, and the extent of the fall varied with the concentration of Acetylcholine. If the fall in blood pressure was abolished by atropine, it was taken to be indicative of Acetylcholine, especially if similar values were obtained by the frog or leech methods.

If curare was used as the blocking agent, exactly the same apparatus and proceedures were involved, except that I-IO,000 curare was incorporated in the perfusion fluid.

In the natural circulation experiments, the curare was

injected intravenously, with collection of the venous effluent for test purposes. Ten per cent trichloracetic acid was employed to precipitate the blood proteins, and then extracted and concentrated before the assay for Acetylcholine was attempted. (Chang and Gaddum, 1933).

RESULTS AND DISCUSSION

In a very brief series of curare experiments, using the cat's tongue and the natural circulation method, our results were as follows:

Table I.

Curare I-IO,000; 0.4 mgs, Eserine Salicylate; IO mg. heparin; Acetylcholine in gamma per c.c. of effluent blood.

Experiment no.	Resting sample	Stimulation	20 min. later
I.	Neg.	0.II	Neg.
2.	0.05	0.I5	Neg.

The above experiments, although giving slightly higher results than those of Dale and his co-workers, still confirm their findings. Although, our results are comparable, our experiments differed in methods of proceedure. We used the natural circulation, with which Dale reported very inconsistent or negative results, as compared to his perfusion method. Because our series is too inadequate, we will not attempt to discuss the significance of our findings. Further, the methods of extraction from whole blood are so involved and complicated, that at best, we consider them to be subject to great error when dealing with biological quantities. This is especially true when the extraction process is as prolonged, and the extracted substance as unstable as Acetylcholine.

The next series of experiments was the most extensive as well as the most important with respect to our original objective; namely, that of attempting to recover Acetylcholine

from venous effluents following motor nerve tetanization of M Magnesium ćurarized" muscle-nerve preparations. Boyd(I932), working with "curarized" tongue preparations, was able to demonstrate a facilitated response to single stimuli after repetitive stimulation. Lubinska (I935), and Brosnan and Boyd (I936), made similar observations using magnesium"curarized" preparations. Although, there were several distinct differences, primarily with time elements and the nature of initial responses, between the magnesium and curare treated preparations, there also were several outstanding similarities.

Since the above results could most easily be explained on the basis of a chemical mediator, additional information was necessary to uphold this contention. Dale performed experiments showing the presence of Acetylcholine in perfusates after stimulation of curarized preparations, and the following tabulation shows results we obtained in similar experiments, but using magnesium as the "curarizing" agent.

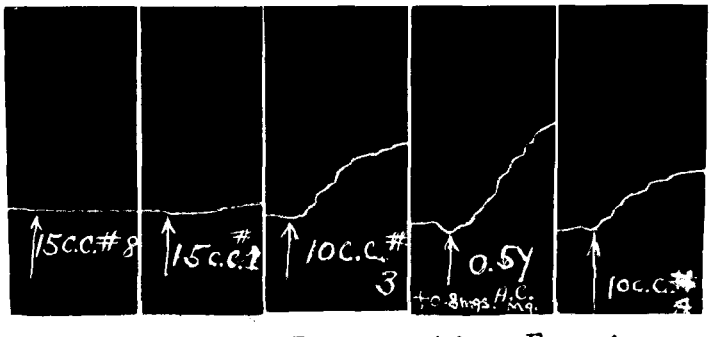

Fig. 1. Leech Preparation.Eserine. $MgSO_4$ (0.8mgs. Mg/c.c.). #8,control, #1,resting. #3 stimulating. #4 following stimulation.

Table 2.

Tongue perfusion experiments, using heparin, eserine, and magnesium sulphate. Mg. concentration--0.8 to I.5 mg./c.c. of perfusion fluid.

Acetylcholine in gamma per c.c. of perfusion fluid.

Exp. No.	During Stim.	After Stim.	During Stim.	After Stim.
I.	0.052	0.02	Neg.	Neg.
2.	.09	.07	.07	.05
3.	.04	.05	Neg.	Neg.
4.	.07	.0I	.04	.03
5.	.06	.03	.03	.0I
6.	.04	.035	.02	Neg.
7.	.I2	.08	Neg.	Neg.
8.	.025	.0I	Neg.	Neg.
9.	.04	.02	.03	.0I
I0.	.035	.025	.0I5	Neg.
II.	.05	.035	.02	.0I

Except for one instance, the initial resting sample always gave a negative test for Acetylcholine, and samples collected during and immediately after a period of tetanization,

always indicated higher Acetylcholine contents. There was a progressive diminution in Acetylcholine in the samples collected thereafter, until samples collected 20 minutes after tetanization were again negative for Acetylcholine. The active substance having either been completely dissipated, or fallen below the threshhold of detectability by biological methods.

Table 2 shows a composite tabulation of Acetylcholine activities obtained in II separate experiments while using magnesium "curarized2 preparations. It shows a fairly regular gradient in activities with a progressive diminution as the perfusion period increases.

For comparative purposes, we ran a short series of experiments identical to the above in all details except that the perfusion fluid was magnesium free. Unlike the magnesium experiments, tetanization of the hypoglossal nerve evoked strong muscle contractions, which often radically altered the rates of perfusion. Although corrections were made on this account, slight discrepancies probably still existed.

Table 3.

Tounge perfusion experiments using Mg.-free Locke's solution. Otherwise conducted similar to experiments of table 2.

Exp. No.	Resting Sample	During Stim.	After Stim.	During 2nd Stim.	After 2nd Stim.
I.	Neg.	0.03	0.02	0.03	Neg.
2.	Neg.	0.045	0.03y	0.03y	Neg.
3.	Neg.	0.043	0.03y	0.02y	Neg.
4.	Neg.	0.035	0.02y	0.01y	0.01
5.	0.009 Y	0.05	0.05y	0.02y	0.01

Barring one experiment in which activity was relatively high, we may say that so far as quantitative comparison is possible, the Acetylcholine recovered in magnesium experiments does not seem to differ perceptibly from perfusates obtained in similar experiments, but using magnesium free solutions. Values obtained varied between .03 gamma and .05 gamma per c.c. of perfusion fluid, which compares rather closely to the magnesium perfusion series (table 2).

Whether the myoneural junction is actually an anatomical discontinuity (Dale), or simply a physiological entity, the magnesium apparently effects its block or terminal depression at this nerve muscle junction. Consequently, nervous impulses coming over a presumably normal nerve trunk, reach the neuro-muscular junction, and although releasing the substance thought by Dale to be essential for transmitting the impulse to the striated muscle cell, the magnesium block, effective at the neuro-muscular junction apparently is responsible for the fai-

lure of the muscle to elicit its customary response.

In this respect, it may be significant to note that when acting directly on striated muscle, magnesium and Acetylcholine have exactly opposite effects. One causes a depression or reversible paralysis, the other a contracture. It is quite feasible to suppose then, that the terminal depression, produced by magnesium, occurs only after its concentration quantitatively surpasses the Acetylcholine concentration.

Figures I and 3 respectively were obtained in specific experiments, but are representative tracings of the muscle reactions in the Acetylcholine assay. They represent results obtained in our perfusion experiments using magnesium as the curarizing agent, but constitute only a fraction of the results listed in table 2.

Figure 3 shows results of an assay for Acetylcholine, using the cat's blood pressure as the test preparation. Here again it is a representative tracing, and includes only one of the results listed in table 2. This method of assay was not used routinely, but only where absolute confirmation of the results with leech and recti tests was deemed necessary.

Because repetitive stimulation, as shown by Boyd, was capable of inducing a prolonged recovery or potentiation of responses to single stimuli, even in the absence of eserine, there was created a situation which could not easily be explained on the basis of the Dale chemical hypothesis. Dale believes that

destruction of the liberated Acetylcholine takes place immediately after liberation, and to that end has performed perfusion experiments using eserine free Locke's solution throughout the experiment. Acetylcholine was not detected in any of the perfusates in these experiments, and the results were explained on the basis of an immediate hydrolysis of the liberated Acetylcholine by a specific enzyme, the cholinesterase of the blood and tissues. The function of the eserine is that of inhibiting the action of the specific esterase on the liberated Acetylcholine.

We have performed a series of slightly modified eserine free perfusion experiments, to which eserine was added within I5-30 seconds after tetanization. This shortened the time interval during which destruction by the cholinesterase could be effective to about I5 seconds. Recovery of Acetylcholine after this time interval, and in the absence of an inhibitor to the destructive agent, would be of great value in explaining the prolonged potentiation described above.

Unfortunately, our results except for one out of six experiments,were entirely negative. This shows that the destructive process apparently occurs well within the I5 seconds allowed before introduction of the eserine an unsolved phenomenon from the standpoint of chemical transmission.

SUMMARY

I. Positive results for Acetylcholine were obtained from cura-

rized preparations when using the natural circulation, but the amounts recovered were slightly higher than in the perfusion experiments.

2. Perfusates collected during and immediately after a period of tetanization of the motor nerve under magnesium narcotization yield the highest concentrations of Acetylcholine (.03 gamma-.05 gamma Acetylcholine per c.c. of perfusion fluid).
3. There is a progressive diminution in the Acetylcholine concentrations, until after I0-20 minutes, the samples are again negative for Acetylcholine.
4. If subjected to a second period of stimulation, there is a sharp rise in the Acetylcholine concentration, but also a very rapid dissipation.
5. Experiments in which magnesium-free Locke's solution was used yield approximately the same concentrations of Acetylcholine, as those in which magnesium curarization was employed.
6. Eserine free experiments in which the anti-esterase is added within I5 seconds after stimulation gives a negative test for Acetylcholine.

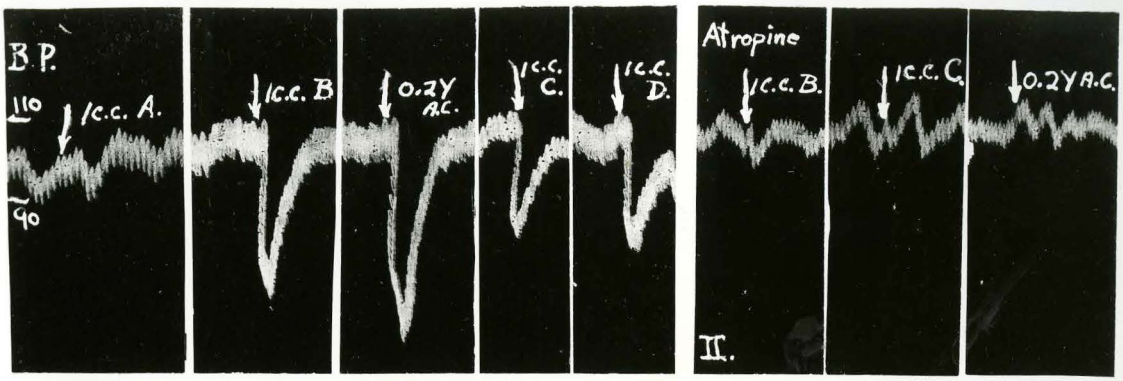

Fig.2. Blood Press. of cat under Nembutal. Eserine 0.2mgs. per kilo. Atropine (1.2mgs.) at II. A, Control. B, During stimulation. C, Immediately after stim. D, Immediately after C. Samples given in 1 C.C. doses (i.v.) and represent 8 c.c. of perfusate. $MgCl_2$ (1 mg. of Mg/c.c. of perfusion fluid.).

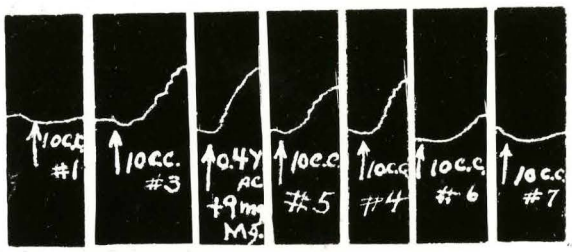

Fig. 3. Frog rectus abdominis. Eserine. $MgSO_4$ (0.8mgs. of Mg per c.c.). #1, resting. #3, stim. #4, Immediately after. #5, after 4. #6, after 3min's. #7, control fluid.

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