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Proceedings of a Conference on Botulinum Toxin
28 and 29 June 1965

edited by

Dr. Charles C. Hassett

July 1966

Medical Research Laboratory
Research Laboratories
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FOREWORD

Poisoning by Clostridium botulinum presents many problems that have been matters of concern to the Medical Laboratories, US Army Edgewood Arsenal Chemical Research and Development Laboratories, for a considerable time. Research on these problems has involved other elements of CRDL and some elements of the US Army Biological Laboratories, Fort Detrick, Maryland, as well as contractors in many nongovernmental research laboratories. This work was performed under Project 1C622401A097, Medical Defense Aspects of Chemical Agents (U).

When many investigators are conducting research on the several aspects of a general problem, it is helpful to bring them together periodically for an exchange of information on a formal basis, with opportunities for informal group and personal discussions. The papers that compose this report represent, therefore, work that has been completed to a stage justifying such a presentation. The discussions following the papers hint at, but cannot capture, the extended and lively exchanges that filled all the free time of the 2-day conference. These discussions may well be the stimuli for future valuable research.

The authors and discussants have read and edited this material in varying degrees, as seemed necessary to expand or clarify the relatively informal presentations that were possible in a conference at which nearly everyone was an expert. In the heat of debate, a few exchanges failed to record well and have been lost. We are greatly indebted to the speakers, who have extended many courtesies in helping to put these proceedings into print.

DIGEST

Twenty monographs were presented at a conference on botulinum toxin held at Edgewood Arsenal on 28 and 29 June 1965. Each presentation was followed by a discussion period. Data in the following fields of research were presented: (1) pharmacological aspects, (2) relation to body weight, (3) dissociation of Type-A toxin, (4) lethal subunits of Type-A toxin, (5) availability of toxin forms, (6) important research questions, (7) possible absorption and distribution sites, (8) localization with lead binding at motor end-plates, (9) isolation of muscle-tissue activity, (10) fractionation and fluorescent labeling, (11) atrophy in chick muscles after massive doses, (12) photodynamic treatment, (13) pharmacodynamics, (14) clinical-pathological studies in monkeys, (15) the use of latex agglutination as a test technique, (16) results of IIT experiments, (17) production of human antibotulism serum, (18) lung absorption of high-molecular-weight molecules, (19) choline-C14 and acetylcholine-C14 distribution, and (20) central and peripheral effects of Type-A toxin. The basis for these studies was a close evaluation of the mechanism of action of the toxin, its effect on tissues, and an improvement in diagnostic and therapeutic techniques.

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5. Dr. Frederick Coulston — Albany Medical College of Union University

6. Dr. Scott V. Covert — Albany Medical College of Union University

7. Dr. F. Crisley — Taft Laboratories, Cincinnati

8. Dr. Daniel Drachman — Tufts University School of Medicine

9. Mr. Leo Feinsilver — Toxicology Division, CRDL

10. Mr. Joseph Fleisher — Physiology Division, CRDL

11. Dr. E. Ross Hart — Bionetics Research Laboratories, Falls Church, Virginia

12. Dr. Charles C. Hassett — Physiology Division, CRDL

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14. Dr. Carl Lamanna — Army Research Office, Arlington, Virginia

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20.	Mr. Samuel Sass	Chemical Research Division, CRDL
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SESSION I

28 June 1965

Chairman: Dr. Ludwig Sternberger
Chief, Pathology Branch, CRDL

INTRODUCTION

Joseph R. Blair, Col, MC
Directorate of Medical Research,
CRDL

Gentlemen, I had hoped to have either Col J. H. Batte, our US Army Edgewood Arsenal Commander, or Dr. S. D. Silver, our Laboratory Director, here to give this welcome, but neither is available. I consider it a privilege and an opportunity to welcome you to Edgewood Arsenal and, more particularly, to the Directorate of Medical Research of CRDL for this confer- ence. I appreciate your leaving your jobs and busy activities at home to spend these 2 days with us. Your presence here will be very profitable to all of us, and we hope that it will be equally profitable for you, as well as a pleasant experience. We have a very fine program that has been arranged and adminis- tered by Dr. Hassett, to whom we give most of the credit for setting up this conference and taking the administrative responsibility for it.

I am pleased that these presentations are unclassified because this will make for a much clearer exchange of information and better discussions. I hope that during the meeting everyone will make comments, ask questions, and conduct extensive informal discussions. The light-blue book that has been sent to most of you will serve as information on the subject. If there were no free exchange of information, there would be very little value in holding this conference, because most of the material could be obtained from these blue books. If we do have a free exchange of information, we can contribute to the mutual programs among our contractors and in our own laboratory; in this way, we can make these programs more responsive to our needs and to our requirements.

Botulinum toxin is a very interesting subject because not only is it of military significance, but it is also a civilian medical problem of some con- sequence. This type of presentation and discussion can be useful to us and to the civilian medical community.

Again, on behalf of both Col Batte and Dr. Silver, I want to wel- come you to this conference and to extend their best wishes, as well as those of the Directorate of Medical Research, for a very successful, profitable, and pleasant conference.

PHARMACOLOGICAL ASPECTS OF BOTULINUM POISONING

Dr. Vernon Brooks
New York Medical College

The basic mode of action of botulinum toxin is well-understood: it puts the neuromuscular junction out of action. It is intriguing that this pharmacological analysis was made about as quickly as the physiological discoveries on how a neuromuscular junction works were made.

At this time, we are going through a cycle of hesitation and confusion about the detailed mode of action of botulinum toxin, and, for this reason, it is justifiable to give a little historical sketch of what we think is true today about this mode of action.

The five types of botulinum toxin all seem to act in the same way. Their antigenic differences are reflected in dose-response relations, but not in their pharmacological mode of action. Type A is the most potent neurotoxin and perhaps the best-studied type. The toxins travel through the body and the bloodstream, and they affect the neuromuscular and autonomic junctions. These structures are functionally similar in that transmission from one neuron to another is mediated by the chemical local hormone and transmitter acetylcholine (ACh). There are two sets of cholinergic transmission points in the autonomic system: the sympathetic ganglia and the peripherally located parasympathetic junctions between cells. Both types of cholinergic junctions are affected by botulinum toxin. In contrast, adrenergic junctions appear to be unaffected. As far as the animal is concerned, death usually results from respiratory paralysis. A good example of the autonomic story is provided in the instance of the eye. The sensory function of the eye is normal in botulinum-poisoned animals, whereas the skeletal neuromuscular junctions and the cholinergically innervated constrictor muscles of the iris are paralyzed. The adrenergically innervated dilators are normal. Other autonomic examples are the pelvic nerves to the bladder and the cholinergic action on the gut, which are both paralyzed. Therefore, we have the botulinum symptoms: dilated pupils, lack of urinary control, constipation, and so forth. The vagal slowing of the heart is least affected. Examples of sympathetic dysfunction are paralysis of the superior cervical ganglion, measured by lack of control over the cat's nictitating membrane.

The main topic here is the neuromuscular junction. Figure 1 shows a spinal motor neuron and its axons, which, in the periphery, branch and subbranch about 100 times. This is the motor unit. Each axon branches to innervate about 100 skeletal-muscle fibers. The action of the different parts will be referred to in turn. There appears to be no explanation for the skeletal-muscle paralysis produced by botulinum toxin in terms of the motor neuron because there is no apparent major dysfunction of the ventral spinal horn cells. Some pathological reflexes can occur, such as the exaggerated H-reflexes reported in the last year or two, but these are minor effects. Myelinated axons are known to be normal. Yet, the poisoned muscle does not contract when the motor nerve is stimulated. In 1923, it was discovered that neuromuscular transmission stops because transmission from nerve to muscle fails. When the motor unit is checked carefully, however, one finds that it does not fail all-or-none, but that it breaks up. The contraction values of a motor unit or its electrical size shows that muscle fibers within a motor unit fail one at a time.

Now we have established the focus of attention; namely, that the botulinum toxin acts somewhere close to an individual neuromuscular junction. This is what we wish to examine. The direct excitability of the muscle fiber is normal. If it is activated, not through its neuromuscular apparatus but by direct electrical stimulation, it will give a normal twitch. Since the transmission from the motor nerve to the skeletal-muscle fiber is effected through the release of ACh and since this material is available, we can squirt it on the muscle fibers. After botulinum poisoning, they are normally excitable electrically but inexcitable through the nerve. When a small amount of ACh is poured on the fibers, they give the normal response (figure 2).

This is the next stage in the analysis of the presynaptic part of the synapse. The unmyelinated little branch called the nerve filament forms a nerve terminal (there is no established term in the literature for this), and there are some final little branches that appear to do the actual releasing of ACh. This may be the area most sensitive to botulinum toxin. This is called the end-plate region in the muscle. The whole is the synapse, the junction between two excitable cells, which in this instance is the nerve and muscle. Everything up to the thin cleft that separates nerve ending from muscle is called the presynaptic part of the apparatus. The receptive substance for ACh and the muscle upon which it lives are called the postsynaptic part of the apparatus. Therefore, the muscle is normally excitable by direct, electrical stimulation or by artificial pouring-on of ACh and not by nerve excitation, which defines the statement that botulinum toxin acts presynaptically somewhere near the nerve endings.
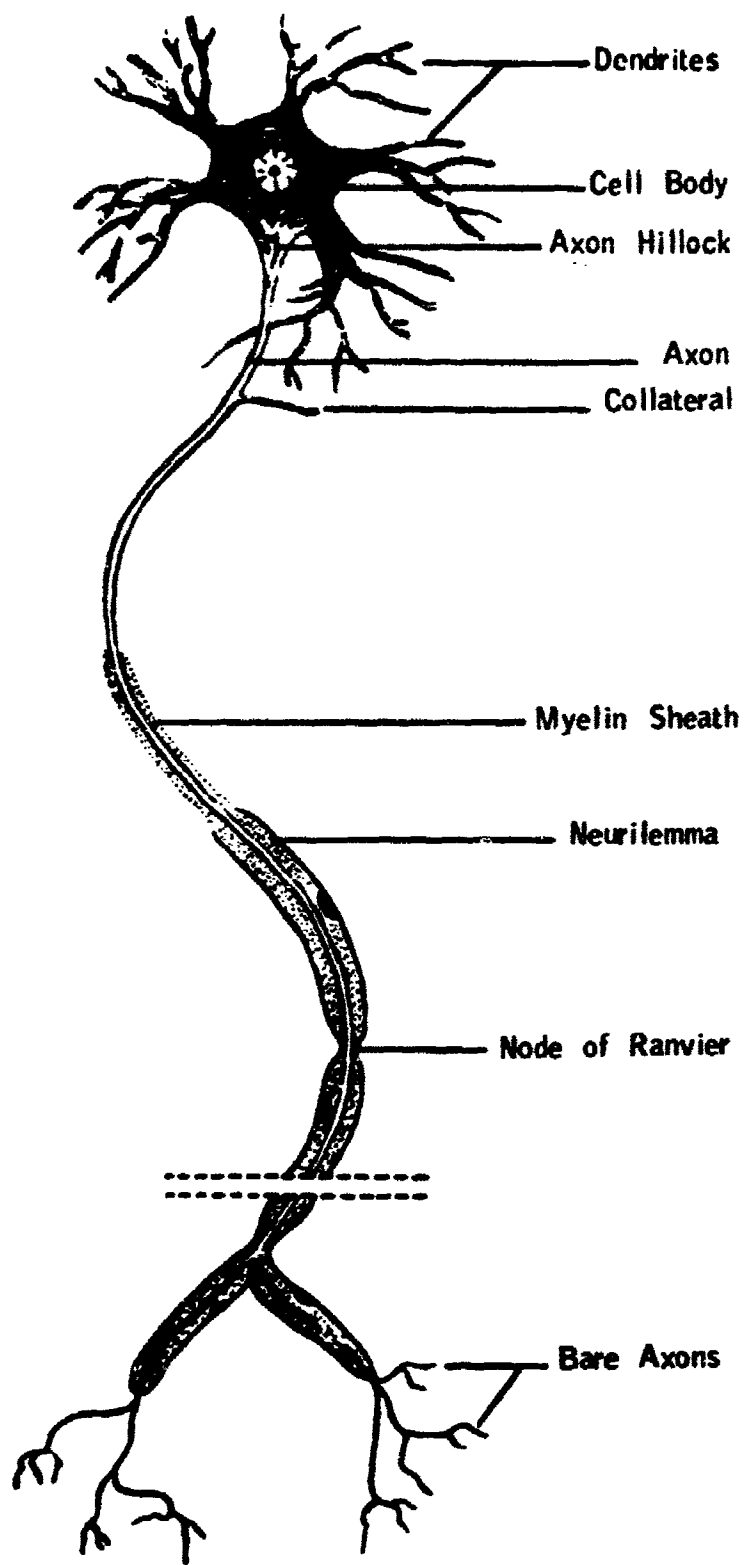
FIGURE 1

SPINAL MOTOR NEURON AND ITS AXONS

FIGURE 2

CLOSE IV INJECTION OF ACh

A. Normal preparation stimulated at six per minute (at arrow, 10 µg of ACh injected rapidly into inferior vena cava)

B. Same preparation after toxin when response to nerve stimulation was reduced to 10% to 15% of initial

C. Same preparation when unresponsive to nerve stimulation

Figure 3 is Arnold Burgen's slide showing the neuromuscular paralysis of the rat's excised diaphragm in a bath; the nerve is stimulated at some reasonable interval (two per minute), resulting in maximal contraction. Botulinum toxin is poured on, but nothing happens for about half an hour. (This inactivity is very important; we do not know what it means, and it will be discussed later.) Then the contraction decreases. If one were to repeat this experiment under close observation, one could see this fractionation of motor units. Finally, upon direct stimulation of the muscle (second arrow), the normal maximal contraction value of the fibers is obtained. Figure 2, also from Burgen's paper, shows that the response of the muscle to exogenous ACh is normal, although the muscle response to ACh that should be coming from the nerve ending is abnormal.

The finding that there is a normal postsynaptic sensitivity to the transmitter agrees with the pharmacological observations that drugs, such as curare, that antagonize the action of ACh by postsynaptic competition have no influence on the course of botulinum-toxin poisoning. Eserine also does not have a significant action. There may be some effects, but they do not interfere with the progress of destruction of action at a neuromuscular junction. The answer to what has gone wrong here has been known for a long time. If the nerve impulse is directed to the end of the nerve fibers and if muscles are reacting normally to the transmitter, then the only thing that could go wrong is that the transmitter is not released. Using the classical methods of Dale or newer methods, this can be checked easily using a nerve-muscle preparation where you can assay the amount of ACh that is released in response to nerve stimulation.

This assay can be made using a slip of diaphragm or some other muscle that is dissected out carefully and put in a Petri dish, with the nerve set up on stimulating electrodes and isolated so that there is no short-circuiting from the Ringer's solution in the Petri dish (figure 4). Then the nerve can be stimulated, and eserine or some other anticholinesterase (antiChE) will still be in the muscle part. A known number of volleys is now put into the neuromuscular junction. The muscle acts as the incidental host of the nerve endings that release the ACh for assay. The direct excitability of the muscle can be checked either by placing stimulating electrodes on it or by placing large metal plates in the bath and passing current through the bath and preparation. There is a difference between these two methods; namely, when the aneural end of the muscle is stimulated, current is sent only through the muscle fibers and they contract. If, however, current pulses are sent through a conducting medium that contains the neuromuscular preparation, current pulses are also sent through the nerve endings (figure 5, N, control).
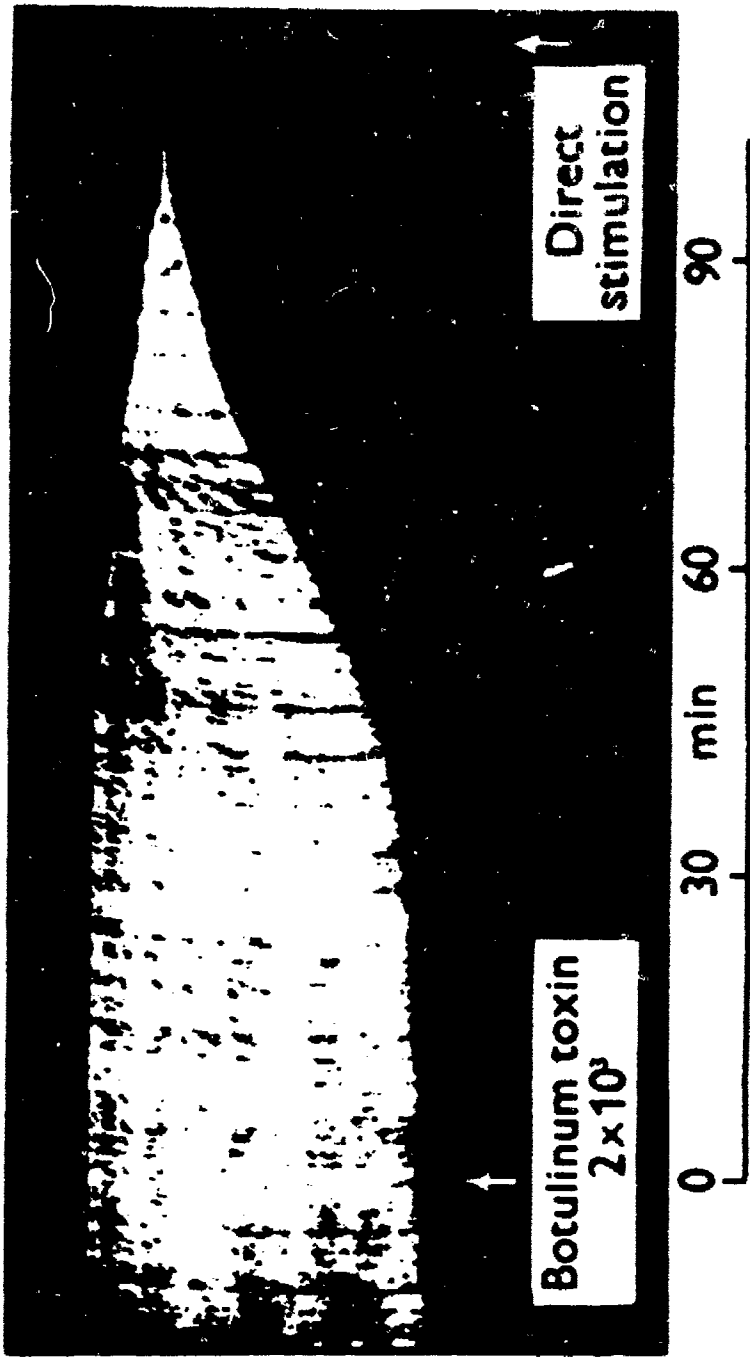
FIGURE 3

NEUROMUSCULAR PARALYSIS OF RAT-DIAPHRAGM PREPARATION AT 37.8°C

(Botulinum toxin, 2×10^3 mouse LD50's/ml, added; normal response to direct electrical stimulation)
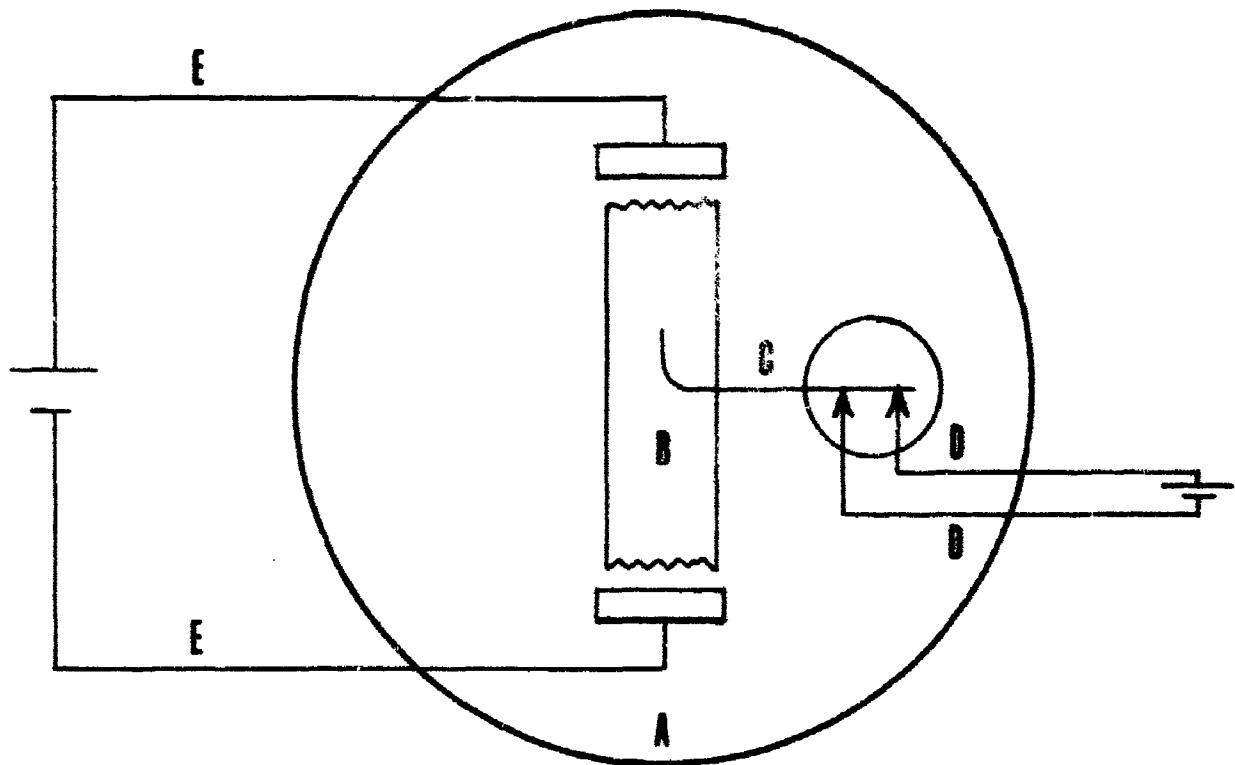
FIGURE 4

APPARATUS USED FOR ASSAY OF ACh RELEASED IN
RESPONSE TO NERVE STIMULATION

A. Petri dish
B. Strip of muscle in saline solution
C. Afferent nerve isolated from solution in dish
D, D. Stimulating electrodes
E, E. Plate electrodes

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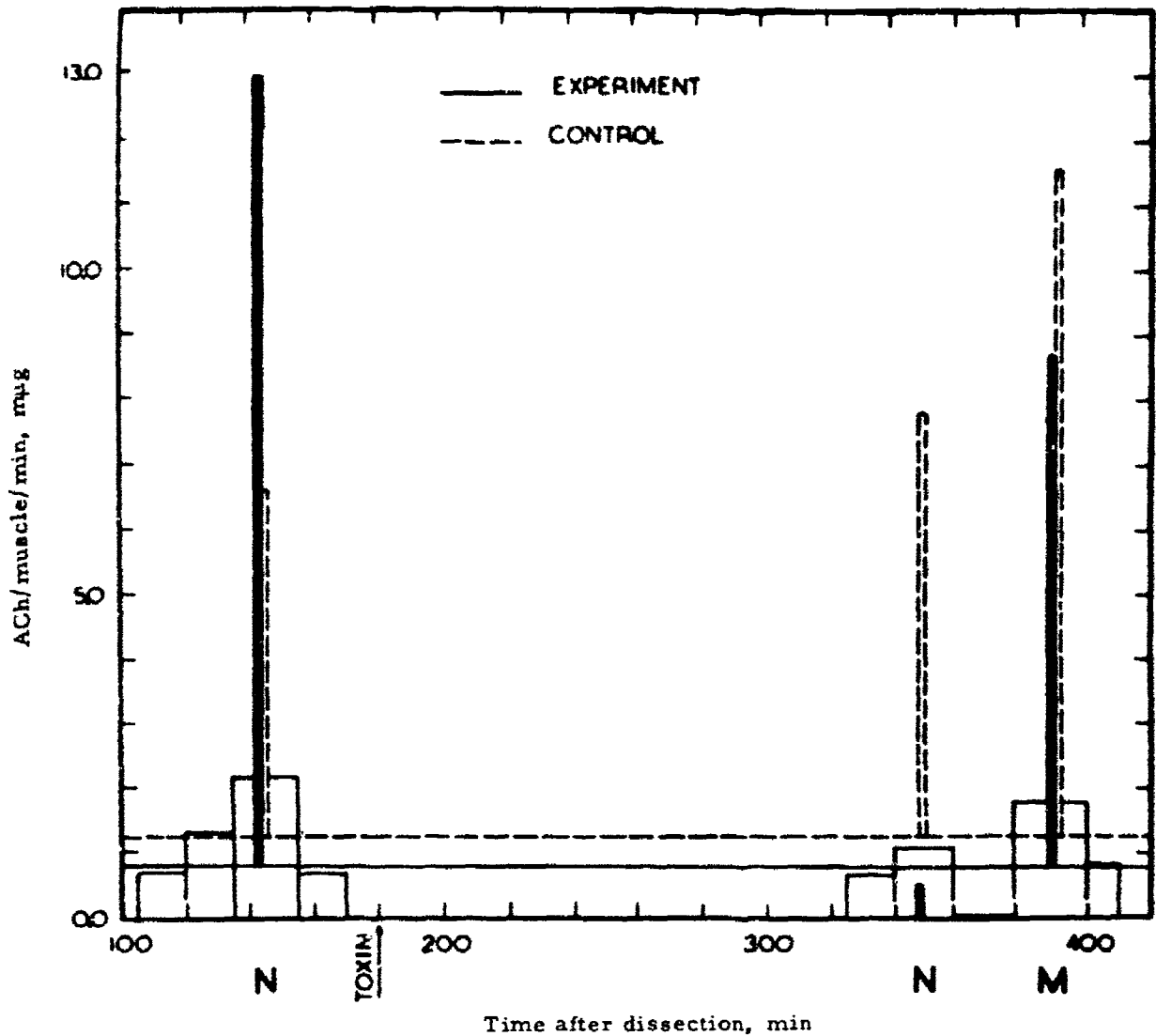
FIGURE 5

RELEASE OF ACh BY MUSCLE BEFORE AND AFTER
POISONING BY BOTULINUM TOXIN

N. Control; stimulation through nerve
M. Stimulation through muscle

This setup was used originally by Rushton to distinguish between the excitability of nerve endings and muscle fibers using curare, which can be used in this type of pharmacological analysis. In the first part of the experiment, the nerve is stimulated, and no ACh comes out after botulinum poisoning (figure 5, N). Then the entire neuromuscular preparation is stimulated, and the normal amount of ACh is released (figure 5, M). This is an old experiment, and the only reason I am discussing it is to reexamine critically what is believed about the locus of action of botulinum toxin. Does it block the nerve endings or just where the ACh is released or the ends where ACh is stored and released?

The experiment in which current pulses went through a bath and ACh was released (although it does not come out in response to stimulation of the motor nerve) suggested that one way of interpreting the locus of action was that the final nerve terminals were blocked off, perhaps by stabilizing the membrane. Nerve impulses stop somewhere at the end, but current pulses go around this block and excite the nerve endings to release ACh. There is a resting level of release of ACh from the preparation that will be discussed later (figure 5, horizontal lines). There is a constant dribbling of ACh that occurs from preparations such as this, and, if the nerve is stimulated, a certain amount of ACh is released; this is shown by the black bar in figure 5. When the preparation is poisoned with botulinum toxin, no ACh is released upon stimulation of the nerve; but, if current is passed through the bath, the normal amount of ACh will come out, roughly within the limits of accuracy of this experiment.

This resting release is not affected substantially by botulinum toxin. It is one of the few cholinogenic effects I know that are not affected by the toxin. The only other one I know is the production of ACh by Lactobacillus. There is, however, another form of resting ACh release that is affected that will be mentioned later.

These experiments suggested that there was a block in the nerve endings. Then the newer methods came along, and we learned what happens at a junction. Instead of assaying ACh in a bath, it became possible to ascertain how much ACh was released by a neuromuscular junction through electrophysiology, by putting a micropipet into the neuromuscular junction and observing the electrical changes on an amplifier. There is a membrane potential between the inside and outside of the fibers that is approximately 70 mv. When a nerve volley comes down and ACh is released, the fibers become short-circuited, and a potential difference of about 0.1 v is generated that then sweeps up and down and is the progenitor of the muscle contraction.

When you observe what happens intracellularly during botulinum poisoning, you see that the generation of postsynaptic electrical potentials is stopped and that there is a constant, very small release of ACh that is electrogenic (not as much as is shown at the baseline of figure 5). There is a constant dribbling of ACh from the nerve ending that causes very small observable electrical discharges. We know from electrophysiological experiments that this small release of ACh comes from the very tip of the nerve.

These electrogenic, electrically visible resting releases of ACh (called miniature end-plate potentials) are stopp.. by botulinum toxin. How they are stopped indicates that they are stopped all-or-none at the source rather than that there is any interference with their reflection as seen through the postsynaptic membrane. There is, however, no electrical counterpart to the much larger dribbling of ACh from the nerve endings seen in the baseline of the other experiment, which was nonelectrogenic. That release is not stopped by botulinum toxin.

It is concluded that botulinum toxin does not act by blocking just the fine nerve terminals, by stabilizing them in some way so that the nerve impulse cannot invade them, but that it is the botulinum toxin located at the site of ACh release that produces the blockade. These little electrical potentials are abolished together with the nerve-induced transmission of impulses through ACh. ACh packets are released right from the endings, somewhere within the gutters, as they are called, and this is as close as we have been able to come to finding out, by pharmacological or physiological methods, where botulinum toxin acts at the neuromuscular junction.

Figure 6 shows the decrease of frequency of miniature end-plate potentials, reflecting the amount of ACh dribbling out from the nerve ending against time. The time of application of botulinum toxin in the lower part of the figure coincides with zero in the upper part. It does not seem to matter what we measure about the detailed action of botulinum toxin, but somehow it takes about 1/2 hr to become manifest. We know from Burgen's experiment of mixing a toxin and an antitoxin, however, that the action does begin much sooner because, during the first 2 or 3 min, the toxin seems to have started on its course, but the action does not become manifest until 1/2 hr later. Presumably, this is our timetable for chemical events that we do not understand.
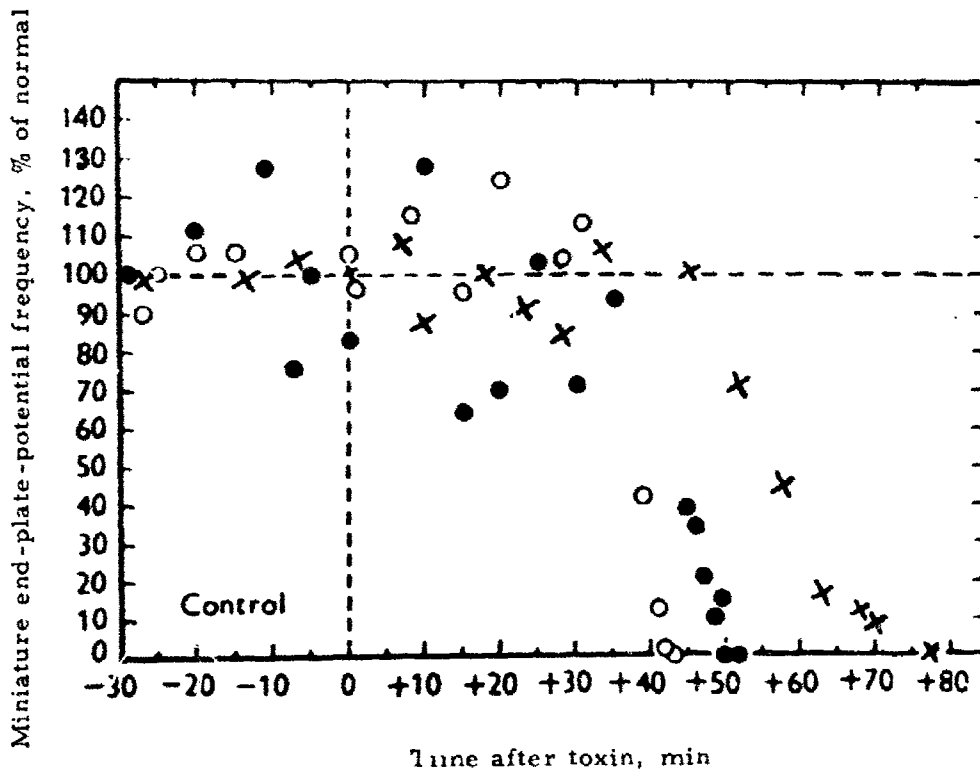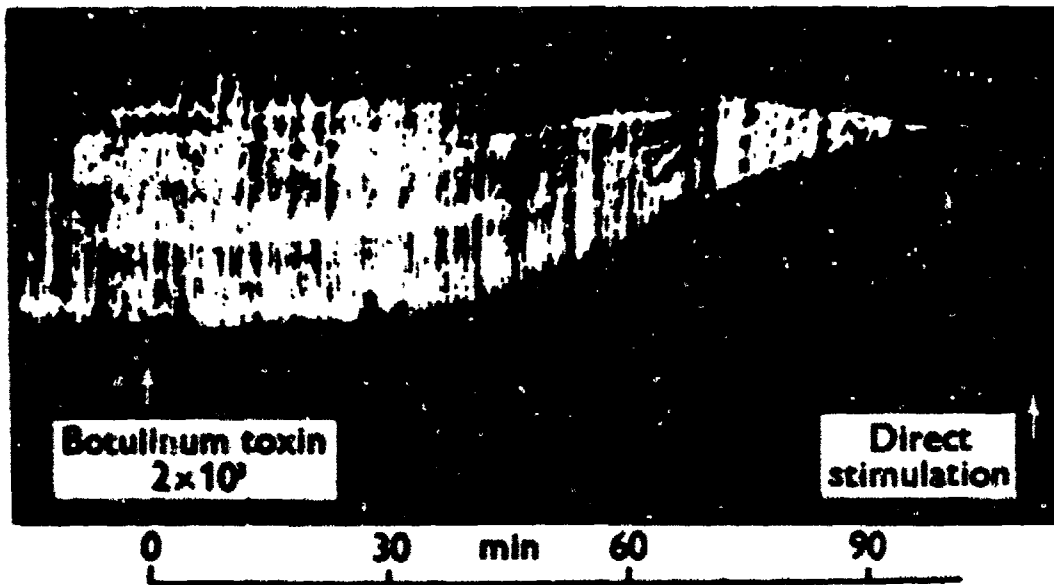
FIGURE 6

RESULTS OF POISONING BY BOTULINUM TOXIN

A. Decrease in contraction of muscle
B. Decrease in frequency of miniature end-plate potentials

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Finally, the miniature end late potentials represent the release of ACh in a physical sense. The electrical changes indicate that there is some ACh being released, because you can do the same pharmacology for these potentials that you can do for ordinary neuromuscular transmission. If you look at the endings of the nerve with the electron microscope, you see in them little packets such as Dr. Coulston mentions (section IV). The packets stain with osmium and are called presynaptic vesicles. They furnish the most likely containers for the transmitter, ACh, that can actually be seen in the nerve endings. An attempt was made to determine by electron microscopy whether, upon botulinum-toxin poisoning, the amount of ACh in the nerve endings decreased in the vesicles or whether the relative positions of the nerve endings shifted. The findings have all been negative. (We cannot do a thing about it because we are not sure enough about the staining methods to know what we are staining.) When the vesicles are counted, which has been done by Katz and Thesleff and in a number of laboratories, there is no reliable, observable proof of decrease.

Just as it seems to take a mysterious 1/2 hr for the action of botulinum toxin to become manifest, it takes a very long time for this action to be reversed. Guyton made animals immune to botulinum toxin by producing local botulism through injections of large amounts of toxin into the muscle. He then had a chronic preparation instead of acute preparations. He found that the recovery of muscular strength after botulinum poisoning took much longer than did the recovery after nerve section or crushing, provided there was reinnervation (figure 7). Some fibers start to recover fairly early, but they are in the minority, and it takes a very long time for the bulk of the fibers to regain their action.

In summary, botulinum toxin is believed to govern the release mechanism of ACh at individual nerve filaments. This accounts for neuromuscular block and for all its pharmacological properties, but the attachment and detachment are not understood at all because of their odd latencies and very long durations.
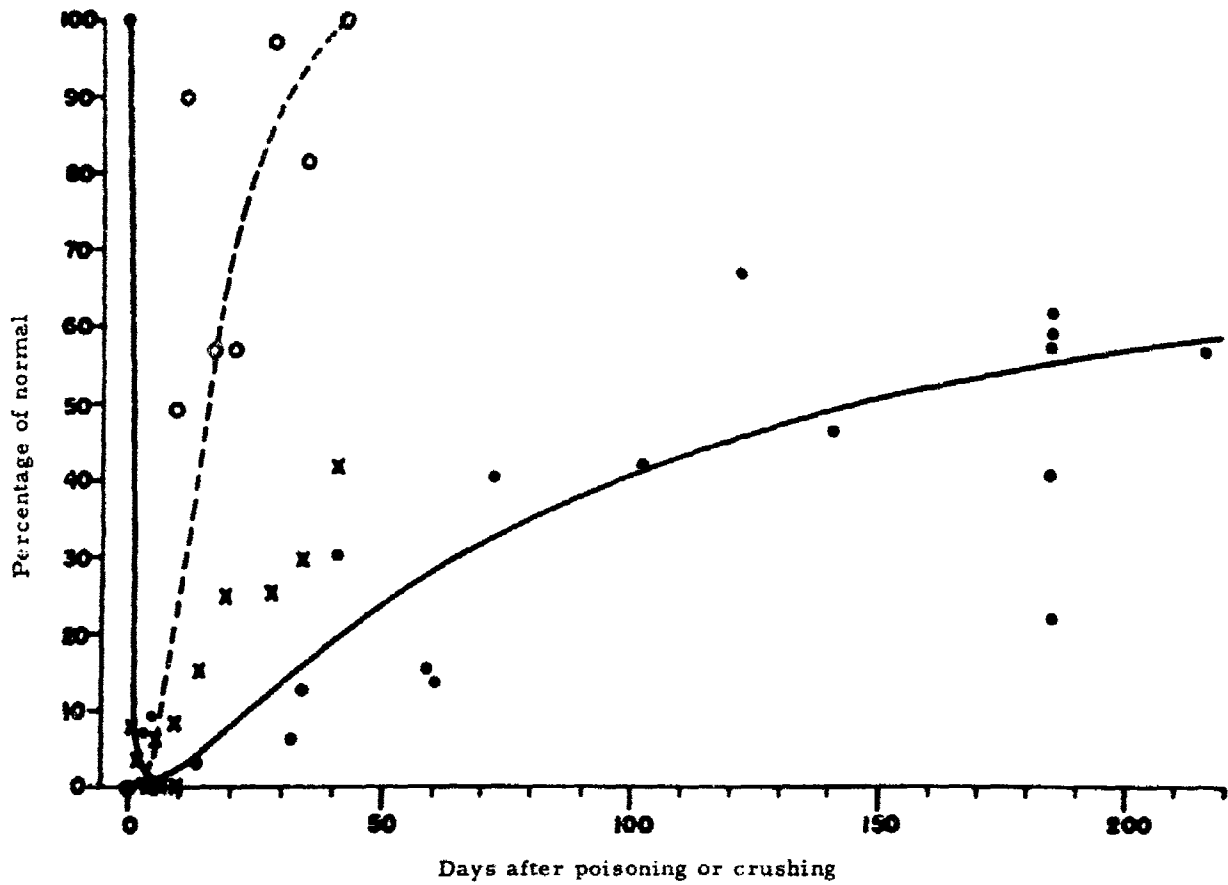
FIGURE 7

RECOVERY OF ACTION POTENTIAL IN GASTROCNEMIUS MUSCLE OF
GUINEA PIGS AFTER BOTULINUM POISONING AND AFTER
CRUSHING SCIATIC NERVE

——— Poisoned prepara⁺ions
- - - - Preparations with crushed nerve
   O Preparations with crushed nerve
●, X Poisoned preparations of two different series

DISCUSSION

Dr. Schantz (Fort Detrick): If the toxin blocks the nerve fibers at the end and prevents liberation of ACh, how is it that when you stimulate at the electrode you get ACh liberation into your bath?

Dr. Brooks: You can have an electrophoretic effect (I don't know what else to call it) of the ACh out of the nerve endings, regardless of the physiological state. It even happens in denervated muscle. I think that it may be a spurious release. I don't know.

Dr. Drachman (Tufts University School of Medicine): What is the evidence that the ACh released in this manner and in large amounts, compared with the miniature end-plate potentials, is released from the nerve ending rather than muscle tissue itself at a distance?

Dr. Brooks: I don't know of any evidence. Maybe I've misunderstood your question. I don't know of any evidence that muscle tissue releases ACh. Would you like to elaborate?

Dr. Drachman: I meant to ask you if there is any hard evidence that this particular ACh is released locally. Denervated muscle will release some ACh if stimulated and, therefore, one would guess that this comes from a similar source.

Dr. Brooks: It is the amount, because you don't get anywhere near that much ACh from denervated muscle. In fact, there is some mysterious continuous generation of electricity observable at the ACh release from denervated muscle that has puzzled people working with it, and they think it comes out of the neural envelope at the endings and so on. That release is also unaffected by botulinum toxin. All these apparently nonneural, unorthodox kinds of ACh releases that we know about are unaffected by botulinum toxin. All that the toxin seems to go after is the ACh that is coming out of the nerve ending, which is a very broad hint indeed as to what kind of an action we are concerned with.

Dr. Lamanna (Army Research Office): I gather then that the release is affected but not the synthesis of ACh. Well then, is it possible to show experimentally that there is an increase in the amount of ACh at those points or reservoirs, or the site of the release where the ACh is normally let out and blocked or goes eventually? Or is there a backup that inhibits the synthesis?

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Dr. Brooks: I don't know. You cannot assay ACh content in nerve endings. You can do that in the electric organ, which in a way is a presynaptic apparatus. The results there are not in conflict with what I've said about the neuromuscular junction. The enzyme that manufactures the ACh is presumably made in the nucleus of the motor neuron and travels down the axon. No such study to check on that has been done so far. Those that could be done or have been done are much like this. Nor do I know if there is any evidence about back effects from release on the system. I don't know what feedbacks are built into this system.

Dr. Coulston (Albany Medical College): Henry Fishback, Chief of Chemistry at the Food and Drug Administration, and I once calculated that to kill a mouse with botulinum toxin, there were approximately 500,000 molecules, for which you estimate 900,000 molecular weight, and I can't conceive how these molecules all fit these end plates. How do these molecules get there? Second, how can we account for so few molecules doing so much damage in an animal, such as lethality in this case?

Dr. Brooks: Well, I suppose the mouse dies when the relatively small number of neuromuscular junctions in the diaphragm have been paralyzed. The rabbit dies when its nares cannot move any more; most other animals die when the diaphragm stops moving. I think there is a considerable possible error in the calculation of the number of molecules. I know it is a fairly small number. Phrenic endings are about as sensitive as any others. I don't know whether today one can say from such a calculation that the mode of action has to be a certain way. If you insist that the number of molecules is too small to go around among the endings, then everything we have said up to now must be wrong. There would have to be some other generative action of the toxin that somehow spreads out and makes a connection with the junction. I don't know, maybe that accounts for the mysterious 1/2 hr, , but I've always assumed that, during this time, attachment of the toxin occurred to the target molecules. It has a high Q_{10} but that doesn't mean much. That is a very relevant point you are raising, Dr. Coulston, but I don't know that one can give a yes-no answer.

Dr. Sternberger (CRDL): I don't think there need be any conflict between your findings of neuromuscular localization of the physiologic lesion and Dr. Coulston's most justified argument. It is true that as little as 1 molecule of toxin per 6 to 50 cells (depending on the mode of introduction) will be lethal. It is indeed difficult to conceive how so few molecules per cell would be able to cause the physiologic lesion by localizing all at the relatively large neuromuscular sites. We cannot assume that a special attraction of toxin to the

neuromuscular unit is responsible for the effect, since intermolecular forces decrease with the seventh power of the intermolecular distance. It is here where Dr. Coulston's concept of the negative phase becomes important. It is conceivable that botulinum toxin acts first on tissues other than the neuromuscular junction, perhaps the endothelium or even intestinal epithelium, and that this interaction secondarily liberates an endogenous substance that is noxious to nerve-muscle transmission. This, also, may explain why antisera lose their effectiveness soon after poisoning. The alternative argument, that antisera lose their effectiveness because of binding of the toxin, is not logical, because it is based on the circular argument that toxin is bound because antisera lose their effectiveness.

<u>Mr. Paul Cresthull (Edgewood Arsenal-ORG)</u>: With reference to this latent period of 1/2 hr, suppose after an organism was poisoned with toxin you added antitoxin within 7 min; would this abolish the reaction of botulism?

<u>Dr. Brooks</u>: Arnold Burgen did that experiment, and the answer is 3 min; after that, no.

<u>Dr. Drachman</u>: The question of activity of toxin for a specific muscle or for individual muscle fibers intrigues me. Have you found that this does not work? Have you any different responses, for example, failure to abolish the end-plate potential in any of your muscle fibers, or is this absolutely universal once the muscle is poisoned?

<u>Dr. Brooks</u>: I think it always happens. I could explain the way the experiments are done to those who do not play about with miniature end-plate potentials. You put a nerve-muscle preparation in a dish, take a very fine glass tube with a conductor in it hooked up to an amplifier, poke it into an end plate, observe the effects, pu'l it out, and go into another one and so on. You can sample during the time that paralysis is occurring, which on the slide was about 1 hr. During that time you can sample quite a few cells, and then thereafter you have as much time as the preparation will allow, so the sampling can include maybe 50 junctions in one preparation that have been poisoned. I've never seen a junction that would still fire nor has anybody else as far as I know. I see no reason why it might not be so, and for what it's worth, I should make the point that it isn't junctions we're talking about but individual terminals. It may be possible to demonstrate, in the normal neuromuscular preparation, differences in the electrically observed release properties of ACh (you know if you put your microelectrode under high power, near different parts, often individual presynaptic apparatus, you will see different things). Although whole junctions seem to go the same way, it might turn out that individual parts that do the

actual releasing will behave differently. I would not expect any categorical difference. One experiment that hasn't been done in connection with botulinum toxin and can now be done, with methods developed by Hubbard largely in Eccles' lab, is to stick microelectrodes into the presynaptic ending.

Dr. Coulston: How thin are the electrodes?

Dr. Brooks: About $1/2\mu$, something like that. You don't see them, but you know they are there because of their resistance.

Dr. Rosenblum (Albany Medical College): I think it has been demonstrated now that if you use a gastrocnemius preparation, you can record from the dorsal root so that you can measure antidromic impulses when you stimulate the muscle or the neuromuscular junction. I think that an experiment of this sort would tell us whether backward or conduction integrity along the axon was still intact. I wonder whether anybody has done that as yet.

Dr. Brooks: Yes.

Dr. Rosenblum: And does it or does it not block the antidromic impulse?

Dr. Brooks: No, it's quite all right.

Dr. Rosenblum: It allows it to conduct?

Dr. Brooks: Yes. The conduction in both directions is all right. What has not been done is the experiment by Stannard, Riker, and Werner, and so on. I know you're referring to those experiments (Dr. Rosenblum: Yes) that are modifications of Masland and Wigton's old experiment. Lloyd studied it. If you depolarize muscle nerve endings, you get antidromic firing in response. That particular induced kind of backfiring has not been studied with botulinum toxin, and those two techniques have not been put together. But the electrically induced backfiring of the nerve in that experiment there works quite well.

Dr. Rosenblum: Riker and his group have essentially said that ACh is unnecessary for neuromuscular transmission in that the miniature end-plate potentials can be generated, but they are generated as a consequence of the orthodromic firing. I think this leaves us with a kind of philosophical debacle if the assumption is correct, because we are concentrating on the assumption

that it's a humoral transmission that's responsible for this whole phenomenon, this is what the toxin is blocking. If, however, it's ephaptic or something of that sort and we can do away with ACh, or perhaps if there is another humoral agent, then we may be focusing attention in the wrong place.

Dr. Brooks: I don't know if it's appropriate to go into too much detail about Dr. Riker's hypothesis. I think that he has shown reproducible and very interesting modifications of the backfiring of the nerve endings, causing antidromic discharge. I do not think that he has shown any evidence that the accepted story of how neuromuscular conduction works is wrong.

THE RELATION BETWEEN BODY WEIGHT
AND BOTULINUM POISONING

Dr. Carl Lamanna
Army Research Office

In reporting the effect of the body weight of an animal on the potency of toxin, the tradition is to express potency as lethality or some other harmful action per kilogram of body weight. In real life, there is simply no such standard thing as a mouse, rat, guinea pig, sheep, horse, elephant, or man with 1 kg of body weight. Therefore, the expressions of toxicity, in terms of this convention, are extrapolations from findings with actual animals, either hypothetically blown-up animals or wizened specimens of the real animals. The extrapolation implies the postulate that some linear relationship exists between body weight and response to the toxic drug. This is probably a false assumption when bacterial toxin is involved, since there is no general rule that permits prediction of the relationship between toxic potency and body weight for different modes of exposure to the botulinum toxin. The harm that befalls the host in the botulinum situation is simply a matter of poisoning, and we need not be concerned with the complicating questions of infection.

In botulism, the biochemical substrate or the anatomic site in the peripheral nervous system of the host affected by the toxin may be, in quantity, independent of the body weight of the poisoned animal. This is, at least, a possible hypothesis. The number of nerve cells in man is fixed at birth and, therefore, does not increase with size and age. One may also infer that an increase in the mass of the nerve cells occurs without an increase in the number of receptor sites per cell for the botulinum toxin, if there is no relationship between body weight and toxic potency. In mice, a lack of relationship between body weight and the quantity of toxin required for a fatal parenteral dose has been found. The weight of toxin required for a fatal dose is the same for the small and large mouse. This finding is not peculiar to botulism. We have also found the same thing to be true for tetanus toxin, and similar reports exist in the literature for Shigella dysentery endotoxin in mice, α-naphthyl-thiourea (ANTU) poisoning in rats, and, most surprisingly, histamine in mice.

Table 1 illustrates some of our results.* In this exercise, two different technicians were used to indicate whether or not it is the technician that is responsible for the results rather than the biological substrate.

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* Lamanna, C., Jensen, W. I., and Bross, I. D. J.. Am. J. Hyg. 62, 21 (1955).

Animal groups of varying weights were injected ip with a particular amount of Type C botulinum toxin that would kill about 50% to 60% of the animals. There is, statistically, no significant difference in the number of animals killed, depending on the weight of the animal. The variation among the technicians is certainly as great as the variation among the different weights of the animals.

TABLE 1

NUMBER OF DEATHS IN GROUPS OF 40 MICE OF VARYING WEIGHT GIVEN IP INJECTIONS OF SAME SOLUTIONS OF TYPE C BOTULINUM TOXIN

Animal weight group	Mortality number			
	Trial 1		Trial 2	
	A*	B*	A	B
gm				
8 - 12	19	24	17	27
10 - 14	26	25	26	27
12 - 16	26	26	27	28
14 - 18	28	28	25	24
16 - 20	24	24	26	26
18 - 22	25	25	26	24
20 - 24	26	22	—	—
22 - 26	15	20	—	—
26 - 30	18	23	10**	—

* Indicates two different technicians used in these experiments.

** Only 20 mice were in this group.

Table 2 records the same kind of information for tetanus toxin.† There are small and large animals, with considerable differences in weights. These differences are so large that the animals are of different physiological age. The animal that weighs 7.6 gm has just finished weaning, compared with a sexually mature, 40-gm animal. When you consider it biologically, there is much more actually involved here than a weight difference.

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† Lamanna, C. Ann. N. Y. Acad. Sci. 88, 1109 (1960).

TABLE 2

IP LD50 OF TETANUS TOXIN SOLUTION WITH
MICE OF VARYING WEIGHT*

Average weight		Technician	LD50	
Small mice	Large mice		Small mice	Large mice
gm				
7.6	39.3	A	1,631,000	1,350,000
9	40	A	189,000	244,000
9.7	40.1	A	364,000	283,000
9.4	39.6	A	389,000	305,000
11	39	A	111,000	55,000
		B	81,000	53,000
9.4	37	A	257,000	259,000
		B	323,000	212,000

* Titration values for small and large mice are not significantly
different, variation being within limits of experimental error.
Method of Pizzi (1950), which permits use of Reed and Muench type
of calculation, was employed for determining standard error of LD50.

The differences between the two columns of LD50's calculated for
particular solutions of the tetanus toxin will show, after statistical analysis,
that the differences observed between the two groups are not significant at
the 5% level. Therefore, with the mice used (two genetically different strains)
and the experiments performed at different times at 5-yr intervals and in
different cities (Baltimore, Maryland, and Oakland, California), there is
concluded to be no apparent effect of the weight of individual mice on the
amount of toxin that it takes to kill them. As a matter of fact, the sex of the
animal may have more influence than the weight; but, again, we did not find
any systematic effects of sex for Types C and A toxins. For a particular
weight group, we could not predict which of the sexes was going to be killed
more frequently than the other, based on one experiment and then trying
another situation.

In contrast to the ip route, potency of botulinum and tetanus
toxin is in rough proportion to the weight of the mouse by the intranasal route.

Table 3 summarizes information on this point for tetanus toxin and Type A botulinum toxin on two different weights of animals.* For these two toxins, there is a relationship between the weight of the animal and the amount of toxin it takes to kill them by intranasal instillation of the toxin (the larger the animal, the larger the intranasal dose must be to kill it). Yet, we cannot say that this is a proof of a relationship between body weight itself and the quantity of toxin required for lethality rather than a fortuitous coincidence. For one reason, the respiratory-tract mucosal tissues of the small mouse might conceivably be more permeable to protein than the tissues of the older mouse. If so, this would permit a more ready passage of bacterial toxin into the general circulation and could account for the results observed. Neither the experience with ip injection nor with intranasal instillation of toxin is repeated upon oral administration of toxin in mice of varying size. Both Type A crystalline botulinum toxin and tetanus toxin were tried, and the youthful mouse required more toxin than did the older, heavier mouse for a lethal dose. Tables 4 and 5 give the results of experiments involving mice that were segregated by sex and weight and exposed to different quantities of toxins by dilution of a particular solution.** If there is any trend, it is for more of the larger animals to die than the smaller animals, given the same amount of toxin; this is true for both tetanus and Type A botulinum toxin.

Common sense might dictate a skeptical attitude toward such a finding, but a fact of anatomy may justify it. The small intestine averages 40 cm in young mice and 57 cm in an old 40-gm mouse. Roughly speaking, there is 50% more intestinal surface area provided for the systemic absorption of toxin in the large mouse than in the small mouse. Since the small and large mice require the same minimum parenteral dose for lethality, the lesser oral dose for the large mouse could reflect merely the greater opportunity for systemic absorption before peristalsis removes the ingested toxin from the bounds of the small intestine, where absorption is most prominent.

In the context of these experiences, a scientifically valid expression of toxicity for various animal species is not really available to us. For the toxins I have worked with, I prefer to compare the amounts of toxin required for fatality in real life, and I make no commitment to the hypothesis that derived from the "per kilogram" theory. The route of injection does make a considerable difference.

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* Lamanna, C. Bacteriol. Rev. 25, 323 (1961).

** Lamanna, C. Ann. N. Y. Acad. Sci. 88, 1109 (1960).

TABLE 3

MORTALITY OF FEMALE MICE OF VARYING WEIGHT UPON INTRANASAL INSTILLATION OF TOXINS

Toxin	Weights of mice			Response to toxin		
	Large	Small	Ratio large: small	Mortality fraction		Ratio small: large*
				Large	Small	
	gm					
Tetanus						
Range	32 - 54	15 - 22	—	—	—	—
Av	40.1	18.2	2.2	32/89	52/89	2.6
SD	4.28	1.32	—	—	—	—
Range	32 - 55	11 - 14	—	—	—	—
Av	41.7	12.2	3.4	19/54	35/60	3.1
SD	4.59	0.92	—	—	—	—
Type A botulinum						
Range	34 - 56	11 - 14	—	—	—	—
Av	42	12.6	3.34	45/60	56/60	Incalculable
SD	3.8	0.68	—	—	—	—
Range	34 - 50	11 - 14	—	—	—	—
Av	40.3	12.1	3.32	13/60	22/60	3.4
SD	4.1	1.02	—	—	—	—
Range	36 - 50	13 - 14	—	—	—	—
Av	42	13.4	3.12	20/60	39/60	3.94
SD	3.06	0.4	—	—	—	—

* Based on LD50 values obtained by titration of given toxin solutions in small and large mice. Values larger than 1 indicate greater potency for small (younger) mice.

TABLE 4

TITRATIONS BY ORAL ROUTE OF TYPE A CRYSTALLINE BOTULINUM TOXIN IN GROUPS OF 20 MICE OF VARYING WEIGHT

Toxin	Experiment 1		Experiment 2		Experiment 3			Experiment 4		
	13 - 15 gm	38 - 40 gm	12 - 14 gm	40 - 43 gm	13 - 14 gm	20 - 22 gm	37 - 39 gm	13 - 14 gm	20 - 22 gm	34 - 36 gm
ml										
0.08	—	—	—	—	20	17	—	12	15	—
0.04	19	20	—	—	17	13	17	5	9	13
0.02	2	20	7	16	2	8	11	1	1	11
0.01	1	9	1	15	8	5	8	0	2	10
0.005	2	5	3	7	1	3	7	1	1	0
0.0025	1	3	1	3	—	—	2	—	—	0
0.00125	—	—	0	2	—	—	—	—	—	—

Mortality number

Note: Difference in deaths between 13- to 14-gm and 20- to 22-gm mice is probably not significant; however, LD50 for largest mice is significantly less (less toxin required for death) than for smaller mice.

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TABLE 5

ORAL TOXICITY OF TETANUS TOXIN FOR GROUPS OF
12 MALE MICE OF VARYING WEIGHT

Mortality number

Toxin	Experiment 1		Experiment 2		Experiment 3	
ml	13 – 14 gm	37 – 39 gm	13 – 14 gm	38 – 40 gm	13 – 14 gm	34 – 35 gm
0.75	—	—	2	10	4	8
0.50	5	6	2	4	5	3
0.25	2	1	0	3	1	4
0.125	0	1	0	4	0	2
0.0625	0	2	—	—	—	—
0.03125	0	0	—	—	—	—

Also, expressing a toxicity per kilogram for the mouse is not really inform-
ative, , and, psychologically, it may induce one to think in terms of a false
assumption. Dr. E. Ross Hart and I have recently done similar kinds of
simple empirical experimentation with the rat. The question we have been
concerned with is how typical these results with mice are when we extend
them to the rat and to mice using other kinds of drugs. Tables 6 and 7 illus-
trate results obtained to date.*

Unfortunately, we did not get the same results with rats that we got
in mice when we did the experiment using an ip dose. In the rat, there is a
correlation between size and the amount of botulinum required to kill the
animal. Although there are sex differences, too, in both male and female
rats, the larger the animal the more toxin it takes to kill it. Table 7 illus-
trates this by showing the LD50 required to kill large and small rats. The
LT50 for a fixed dose is also recorded (table 6). This, too, differs from that
with-mice, with which we were able to show that both the LD50 and the length
of time it took to kill the mouse were not affected by the weight of the animal.
This was not repeated in our experience with the rat.

In going through the literature, Dr. Hart found that there was a
scarcity of published material on the weight of the animal and its influence on
the toxicity of many commonly used materials in the laboratory. Therefore,
we chose the compounds listed in table 7 and determined the toxicity for both
male and female, in terms of any influence of weight. In every single com-
pound that we tried, there was an effect of body weight on the potency of the
compound. This is a diverse group of compounds in terms of the physiological
nature of the mechanism that is killing the animal. We have compounds that
affect the nervous system in different ways and some that are cytoplasmic
poisons. We also have had an unfortunate result in our experiences with
histamine. A report by Angelakos** states that, in terms of lethality, the
potency of histamine for the mouse is independent of body weight. In our
results, this finding has not been confirmed, because the larger mice require
one and one-half to two times as much histamine to be killed, depending on
whether the animal is male or female.

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* Lamanna, C., and Hart, E. R. Unpublished data.

** Angelakos, E. T. Proc. Soc. Exptl. Biol. Med. 103, 296 (1960).

TABLE 6

INFLUENCE OF BODY WEIGHT ON TOXICITY OF BOTULINUM TOXIN INJECTED IP INTO RATS

Size and sex	LD50	LT50*
	$ml \times 10^{-5}$	hr
Large, male	9.00	47.3
Large, female	8.18	40.2
Small, male	5.00	22.8
Small, female	3.64	24.6

* Dilution, 3.1×10^{-5}, of a given solution of toxin.

TABLE 7

INFLUENCE OF BODY WEIGHT ON TOXICITY OF VARIOUS DRUGS INJECTED IP INTO MICE

Compound	Molecular weight	LD50 ratios for 26-gm:10-gm mice	
		Male	Female
		moles	
Botulinum toxin	100,000	1.0	1.0
Atropine sulfate	695	2.36	2.12
Hemicholinium-33	575	2.06	1.23
Histamine	307	2.23	1.67
Sodium pentobarbital	248	2.61	2.85
Sodium barbital	206	2.67	2.45*
ANTU	202	7.45	7.71
Sodium fluoroacetate	100	1.97	1.79

* Data incomplete.

We chose hemicholinium because, in reading the literature, we felt that this drug might have nearly the same kind of action as botulinum toxin. It, too, interferes in some way with the release of ACh. With mice, however, we did not duplicate the results we obtained with the botulinum and tetanus toxins. In table 7, the number 2.06 means that the ratio of LD50 in moles per mouse is weight-dependent. That almost corresponds to the difference in weight of the animal, since we are comparing 26-gm with 10-gm mice. Therefore, hemicholinium, as a drug that might appear superficially to have the same general type of action as botulinum, does not act like botulinum toxin in the mouse. We would be happy to receive suggestions for other drugs to test.

All of our results are expressed in moles because, scientifically, the weight of a material is not as important as the number of molecules the animal receives. Relating toxicity and weight involves not only questions of the route of injection, but also of the species of animal. It is very difficult to predict from one drug what the situation is going to be when you try another drug. At present, we are in the unfortunate position of not being able to make any generalizations for us to memorize and apply, by rote, in the future.

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DISCUSSION

Dr. Stemmer (University of Cincinnati): Was there a time relationship between death and application of the toxin in regard to whether the large animals died faster or slower?

Dr. Lamanna: With botulinum toxin in the mouse, there was no difference in the length of time it took to kill the animal, so it was independent of the weight. Neither the LD50 nor the lethal time was affected by the weight of the mouse with botulinum toxin; that is, with Types A and C. We have no experience with the other types. We picked these two, not only because they are available, but because, in nature, Type C has generally had a different type of geographic distribution and presents a veterinary problem, whereas Type A has been the one most important for human botulism in the US.

Dr. Coulston (Albany Medical College): Would you care to discuss how parenteral routes of injection compare to oral administration? Is there any information for that?

Dr. Lamanna: No. We have limited ourselves to only the parenteral ip routes. We have some iv work, too, but this would not affect anything I have said.

Dr. Coulston: Depending on how soon these animals die (I do not know the relationship), there is a well-known phenomenon in toxicology (for example, with rats) that if you put a drug in the food and allow small rats to eat it, when the rats grow up you find that, contrary to what you think, the small rats are getting a lot more of the drug than do the adults. If the animals survive for any length of time, this is a characteristic of feeding that you have to bring into the picture.

Dr. Lamanna: We get away from all problems of feeding in that any oral experiments that we have done are by direct instillation. I don't like to use the word "injection" because we are not penetrating any tissues. We have done autopsies to make sure there is no trauma in the esophagus and the stomach, but exposure is by direct instillation with a blunt-ended needle. In the mouse we have never given, in one instillation, more than 0.25 ml, which we believe can be tolerated by the mice without any trauma. Therefore, in our experiments we have not used a feeding approach to the oral-toxicity situation.

Dr. Coulston: Giving an ip injection is fine, but the animal has to eat, assuming that it is not knocked out completely, and the amount of food it might take would change recycling of many of these compounds and, thus, the results.

Dr. Lamanna: I did not want to talk about the general problem of poisoning by the oral route; I just wanted to focus here on the body-weight business. The kind of food the animal had will determine the magnitude of the fatal oral dose. We have shown this and done some work, not with ethyl alcohol, but with brandy. We have come to a somewhat different conclusion than that in the brown book. In fact, there is no virtue in drinking brandy if you want to save yourself from dying from botulinum poisoning. To do it, you have to drink enough brandy to kill yourself by ethyl alcohol intoxication. We figure that the cure might be pleasant but equally fatal.

Dr. Sim (CRDL): The results obtained from studies in rhesus and squirrel monkeys and chimpanzees were iv LD50's of 55, 70, and 30 mouse units/kg (MU/kg). Average weights of these groups were 3.2, 0.4, and 12 kg. The squirrel monkeys were the only adult population, both rhesus and chimpanzee being adolescent groups.

One word of caution about ip injections. Several years ago, Dr. H. Innes tested the efficacy of using this route of administration. His findings were that approximately 30% of rodents injected by usual laboratory techniques were injected in the gut rather than into the abdominal cavity. Unless such injections are checked frequently by using tracer dyes, a greater degree of variability in results must be anticipated.

Dr. Lamanna: I would like to emphasize that what I was talking about are comparisons of body weight within a species. It is dangerous and foolhardy to extrapolate from one species to another. For example, only last year, experimenters who tried LSD-25 on elephants killed them because they extrapolated from a small rodentlike beast to an entirely different kind of beast like an elephant. Therefore, when I am talking about body weight and potency, I am speaking in terms of comparison within species, and I do not know of any generalizations that would permit you to extrapolate to another species. I think the only test is to try it, because, in my experience, what you do with one species never gives you much predictive ability about how the next species will react.

All of the botulism and tetanus work done by neurophysiologists has been done on animals other than mice. I am very curious about what differences, if any, one might find if one compared in vitro neuroelectro-physiological preparations from mice with rats, assuming that this difference of influence of body weight holds. Theoretically, there is an unchanging number of nerve cells, and once the animal is born and grows up to be a sexually mature animal, then anything we find in regard to effects of weight

cannot have anything to do with the mere number of cells. Yet, something in the nervous system is changing while the animal is growing up and increasing his weight. For one thing the animal is experiencing an increase in the mass of the nervous system. Obviously, for botulinum toxin with mice, if these results are valid, there is no relationship between the mass of the nervous tissue and the effect of the toxin. With the rat, there must be. What can this difference be? If, at the nerve endings, where the ACh is being released, a growth in mass does or does not result in an increase in the number of excretory sites for ACh (if you want to use that term "excretory"), I can visualize a situation where, if you have a 10-gm mouse, he may have two sites that release ACh where botulinum toxin is able to act. As the animal grows up, there could be an increase in mass that merely separates these sites by a greater distance or keeps them together. That could be one possibility. Or, there could be an increase in mass and in number of "excretory" sites in the rat.

If differences in the effects of body weight are due to an anatomic difference rather than some other factor of a biochemical nature, it might be interesting to compare rats and mice by doing the same types of neurophysiological experimentation. I think two possibilities have to be considered. One is that in mice there is an increase in mass without a concomitant increase in receptor sites for botulinum toxin, which is not true for the rat. If there is other than an anatomic explanation, we must seek a biochemical one. In this instance, there is the unexplored possibility that depends on whether the neurophysiologists finally accept Koelle's ideas on the release of ACh. This notion is that a little amount of ACh is released that then acts, as I understand his concept, to stimulate the nerve ending to release still more ACh, which is the actual ACh that acts as a transmitter substance. One could question as to whether botulinum toxin affects the initial site of release or this rerelease and whether there is any difference in the number of one or the other of these sites, depending on the species and on the age (weight) of the animal. I realize, however, that Koelle's material is controversial, and I do not know of anyone who has attempted to see whether botulinum toxin affects specifically one or the other of these mechanisms of ACh release that Koelle speaks about.

Dr. Sim: On your last slide, botulinum toxin was indicated to have a molecular weight of 100,000. Was that an error, or do you have reason to believe this is more correct than 900,000?

Dr. Lamanna: No, we do not. We had to choose an arbitrary figure, and since there are now in the literature claims of everything from 12,000 to 900,000, 100,000 is not a bad guess.

Dr. Sternberger (CRDL): Then you mean 100,000 is pretty close?

Dr. Lamanna: Well, you are dealing here with a material, botulinum toxin, that is so much larger than all these other things, such as hemicholinium and histamine, that I think manipulating a few numbers is not going to change your concept.

Dr. Schantz (Fort Detrick): I think that is a good explanation. At Fort Detrick, we never know the number of molecules, and from your work at California, I think you estimated that the molecular weight of the toxin in the bloodstream was about like that given, which would be very high. We have found that when you dilute the toxin greatly, however, it must dissociate into smaller molecules, so that you would increase the number of molecules as it became more dilute. This could affect the dose that you would give and might be an explanation of the point raised by Dr. Brooks that there are not enough molecules to go around. In dissociation of the large molecules into smaller ones, you might have a greater number of molecules that would reach more sites. I am not saying that specific toxicity would be increased by this, but you could spread the toxin over more sites in the animal body.

Dr. Tyler (Peter Brent Brigham Hospital): Is that about 7, compared to 2.5 for all the others on that last slide you showed?

Dr. Lamanna: This is suspicious, because, again, this contradicts the literature to the effect that ANTU was a compound that acted on the rat independent of the body weight of the rat.

Dr. Tyler: This is a highly significant relationship?

Dr. Lamanna: We cannot explain—we do know that our rats are of a different strain than those used by Rall and North.* Accepting the convention of expressing potency in terms of kilograms of body weight seems to inhibit people from doing any real work on a particular drug to establish what the true relationship is.

Dr. Tyler: When you injected the doses, did you set a time limit to estimate lethality or did you keep increasing the dose so that they all died within a certain period of time?

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* Rall, D. P., and North, W. C. Proc. Soc. Exptl. Biol. Med. 83, 825 (1953).

Dr. Hart (Bionectics Research Laboratories): These data are for acute
actions. All the rest of this is based on the administration of a single dose.
All the injections were made within, generally speaking, 2 hr in every animal
that was injected and then repeated some time later. I do not believe there is
enough difference in time of death to support the contention, and I do not have
the exact data before me.

Dr. Tyler: I am wondering whether you determined the LD50's of all the other
substances by setting up a time in which you wanted the experiment to be com-
pleted? Of course, with phenobarbital you can inject the animal so that he will
sleep and die of pneumonia, but he will indeed die, or you can kill him within
1 hr. I wondered whether you had arbitrarily kept increasing the dose until
they all were sacrificed or killed with the acute LD50 within the time limit.
Were all the experiments, say, 2 or 8 hr?

Dr. Hart: Practically all of it is based on overnight deaths, within the first
24 hr. I do not believe there is enough variation here to bring out anything
along the lines you are suggesting.

Dr. Lamanna: Most of the deaths are grouped over a much shorter interval
of time, although observations are extended overnight. The one difference
between the tetanus and botulinum toxins and these other drugs is that you have
a long incubation phase with the toxins, whereas the drugs cause acute deaths.
Some of them happen within 1 hr after injection, depending on the drug. Again,
we cannot say that the botulinum toxin difference is due to this fact, because
the situation with the rat was different where there was a long incubation.
You do not kill the rat within a couple of hours after injection of approximately
the LD50 dose. Those observations extended for 5 days. Again, most of the
deaths were within 12 to 48 hr.

Dr. Tyler: Judging from human material, I do not think Dr. Lamanna's con-
clusions that the number of nerve cells remains steady with age holds, because
we know that at least in human beings the number of nerve cells decreases
steadily as the animal gets older. What does increase with maturity is the
dendritic growth and complexity of the individual neurons.

      I would like to ask if a 10-gm mouse and a 26-gm rat can be
compared in terms of equal maturity as measured by lifespan? Do we hit
them at the same time? Is body weight the best indication?

Dr. Lamanna: In our experiments, the rat would have a lesser span of physiological age than the mouse. The normal small rat we used would be more mature than the smaller mouse.

Dr. Sternberger: Amplifying on the point that Dr. Tyler raised, Dr. Petty has obtained a great volume of evidence on toxin susceptibility in man. I wonder whether a relationship similar to that found by Dr. Lamanna can be established in man. If such a relationship holds, young people should be relatively more resistant to toxin than old people. Do you find frequent toxicity of botulism in children?

Dr. Petty (Maryland Medical-Legal Foundation, Inc.): This ties in with something I have been wondering about. Your range in body weight in mice represents the extreme small end of the scale, does it not? You have no animals (not mice) that approximate the weight of mice?

Dr. Lamanna: We had some in the intermediate weight group.

Dr. Petty: You had an intermediate weight group, but this is the small end of the weight spectrum, and I am wondering about the results if you used animals of greater weights.

Dr. Lamanna: In table 1, I think there are about eight weight groups.

Dr. Petty: You misunderstand me, I think. You have no animals that are as critical in size as the mice. You run from 8 to 30 gm, but here you have no similarly sized animals of another species. Have you done any work with another animal of this approximate size? I wonder what the effect of the size of the animal would be on the metabolic activity of that animal and, therefore, on the rapidity of dispersal of the toxin to the site at which it acts. I am wondering if there is a much smaller difference between the 8-gm mouse and its metabolic level of activity and the 30-gm mouse and its metabolic level of activity than there is between the rat of the smallest size and the largest size. Do you see my point?

Dr. Lamanna: Yes, but I cannot answer it. You are asking a question here that relates to trying to explain the difference in the species.

Dr. Petty: No, not necessarily in species. I am trying to find out what the difference in metabolic activity between your smallest small animal and the largest small animal would be and the difference between the smallest large animal and the largest large animal would be. In other words, I am trying to find out if there is a greater variation between animals of the large size than there would be between animals of the small size insofar as metabolism, rate of absorption, rate of circulation, and rate of activity by the poison are concerned.

Dr. Lamanna: You are asking about the variability within each of the populations.

Dr. Petty: That is right.

Dr. Lamanna: I have the data for death times for the Type C toxin, but it has been so long ago that I cannot answer your question offhand; my recollection is, however, that in the case of the Type C toxin with the mice, the variability within the population was not significantly different. In other words, the magnitude of variation in which the individual animals died was not significantly different among these groups.

Dr. Petty: I wonder if we should not take another species of animal with the same general size and same spread between its large and small animals and see if this also holds true for botulinum toxin.

Question: He is using the rat?

Dr. Petty: Yes, but that is a differently sized animal.

Dr. Lamanna: Well, inherently, a rat is a larger animal than a mouse. That is what he is saying.

Dr. Brooks (New York Medical College): I do not quite understand. If you are talking about the smaller animal and the metabolic rate, what about the same animal at different temperatures? Then it would depend, as it was pointed out, on how you measure the toxicity. Do you wait long enough for them to die or are you waiting for just a fixed length of time, because previous work on tetanus toxin in frogs showed that as you cool them off, you have to wait longer? I wonder whether it would not be an easier experiment to answer your question by simply taking mice at different temperatures.

DISSOCIATION OF TYPE A CLOSTRIDIUM BOTULINUM TOXIN

Dr. W. H. Riesen
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The studies that will be summarized were performed in a program sponsored by CRDL from 15 September 1960 to 30 April 1964. The program was monitored by Mr. B. Currie and Dr. C. A. Broomfield of CRDL, and the work was done in collaboration with Mr. A. M. Gross. We are indebted to Dr. E. J. Schantz for supplying the crystalline toxin and for his interest and counsel.

The previous paper and all the papers of this session indicate the importance of knowing the size of the botulinum molecule when it intoxicates the organism. The serological Types A through F of botulinum toxin have similar modes of pharmacological action but different molecular sizes and, also, different times to onset of symptoms in all species. A crucial factor is whether the larger molecules dissociate in vivo or are cleaved by the digestive enzymes to smaller toxic units.

The recent Canadian publication by Julia Gerwing and Dr. Dolman and their associates, which reports the direct dissociation from bacterial broth of Type A botulinum toxin to small toxic subunits (molecular weight approximately 12,000), is exciting news. The results of Dr. King's research at IITRI parallel the recent Canadian findings in many respects.

The objective of our studies was to develop methods of producing toxic fragments of botulinum Type A toxin. The important criteria were reduction of molecular size and retention of considerable toxicity. High yield was considered desirable, but secondary, in the initial phase of the program. The approaches included attempts to dissociate secondary linkages in the toxin by ionic shifts or by using guanidine, detergents, and cold mineral acid and attempts to cleave primary peptide bonds by chemical oxidation or by using proteolytic enzymes. Several of these approaches provided evidence of dissociation to units of smaller molecular size and varied toxicity, yield, and stability.

An interesting instance of conformational change and apparent dissociation of the toxin molecule occurred during storage for 1 yr at 40°C in 0.05 M acetate buffer at pH 3.8. Gel filtration of the stored toxin through Sephadex G-75 and elution with water produced a family of peaks that were not only retarded in the effluent but were also altered in their 260:278-mµ absorption ratio (figure 8). Repeated gel filtration of some of the retarded peaks produced partial reversion to the unretarded peak (T) at one void volume.

Second crop toxin stored 1 yr
in 0.05 M acetate pH 3.8
Eluted with water
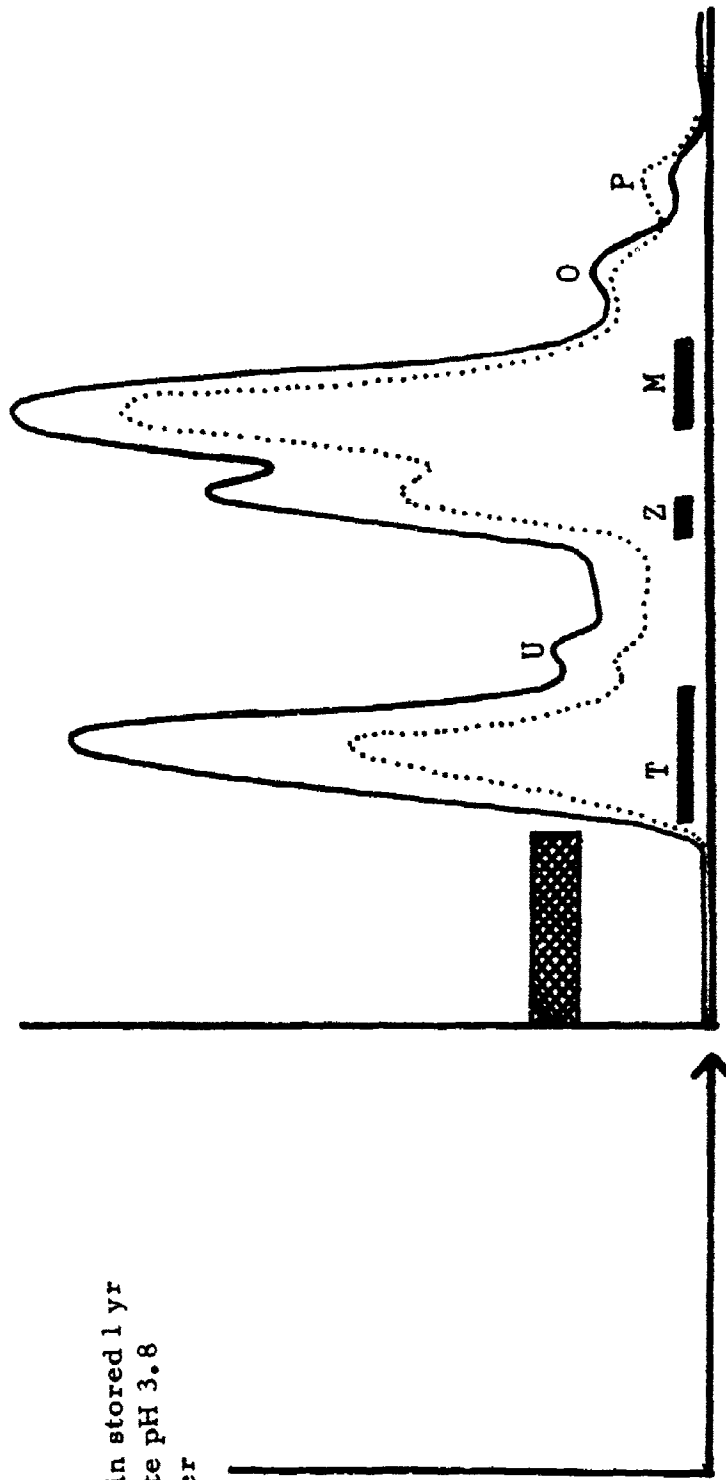
FIGURE 8

SEPHADEX G-75 FRACTIONATION OF BOTULINUM TOXIN BY
WATER ELUTION AFTER STORAGE FOR 1 YR AT 4°C

(Centrifuged at 2,000 rpm to remove sediment)

Cross hatch = column void volume

———————— = 278-mμ absorption

··········· = 260-mμ absorption

This experiment suggests reversible dissociation of the toxin merely by its being aged in acetate buffer. The largest retarded peaks (T and M) resemble those obtained by treatment with cold HCl followed by gel filtration in phosphate. This procedure will be described later.

An indication that high salt concentration favors dissociation to toxic subunits was found in the next experiment (figure 9). Toxin that was stored for 7 days at $4^{\circ}C$ in saturated 0.4 M phosphate-4.0 M sodium chloride at pH 6.8 was eluted from Sephadex G-75 with dilute phosphate (0.05 M) and urea (0.05 M) at the same pH. (The urea was unessential.) The retarded peak was more toxic per unit at 278 $m\mu$ absorbance, according to time-to-death mouse assay, than the nonretarded toxin or the original toxin.

Retardation in a Sephadex column is not proof of reduction of molecular size. In both examples, the column sample was applied in more salt than was present in the effluent. Hence, retardation in the column may be due to a salting-out effect. Such salt interaction, however, is possibly favorable to molecular dissociation. Attempts to dialyze smaller units from the mixture generally were unsuccessful.

Several additional methods of treatment prior to gel filtration with Sephadex G-75 were attempted to separate any dissociated fragments. The treatments included sodium lauryl sulfate, quaternary detergent, phenanthridine hydrochloride, sodium deoxycholate, formic acid, acetic acid, and sonification. None was successful.

Toxin in 0.05 M phosphate buffer at pH 6.8 applied to Sephadex G-75 columns and eluted with the same buffer appeared as one nonretarded peak in the effluent. Stability of the toxin at low pH prompted investigation of mineral acid as a dissociating agent. Treatment with 0.1 N HCl at $4^{\circ}C$ and gel filtration with Sephadex G-75 in the same acid at the same temperature produced no dissociated units. Treatment in the cold followed by application of the acidic sample to Sephadex G-75 columns packed in 0.05 M phosphate at pH 6.8 and elution with the phosphate did produce retarded peaks (figure 10).

In figure 10, apparently undissociated toxin is shown at position T (one void volume), whereas a retarded moiety is shown at positions N and M (two to three void volumes). It was not definitely established in this experiment whether the retarded moiety contained dissociated subunits. Both the T and M peaks had toxicity, however, according to mouse bioassay. The retarded peaks had a higher 260:278-$m\mu$ ratio than the nonretarded peak of the original toxin. The conditions of treatment were 0.1 N HCl at $4^{\circ}C$ for 1 wk.
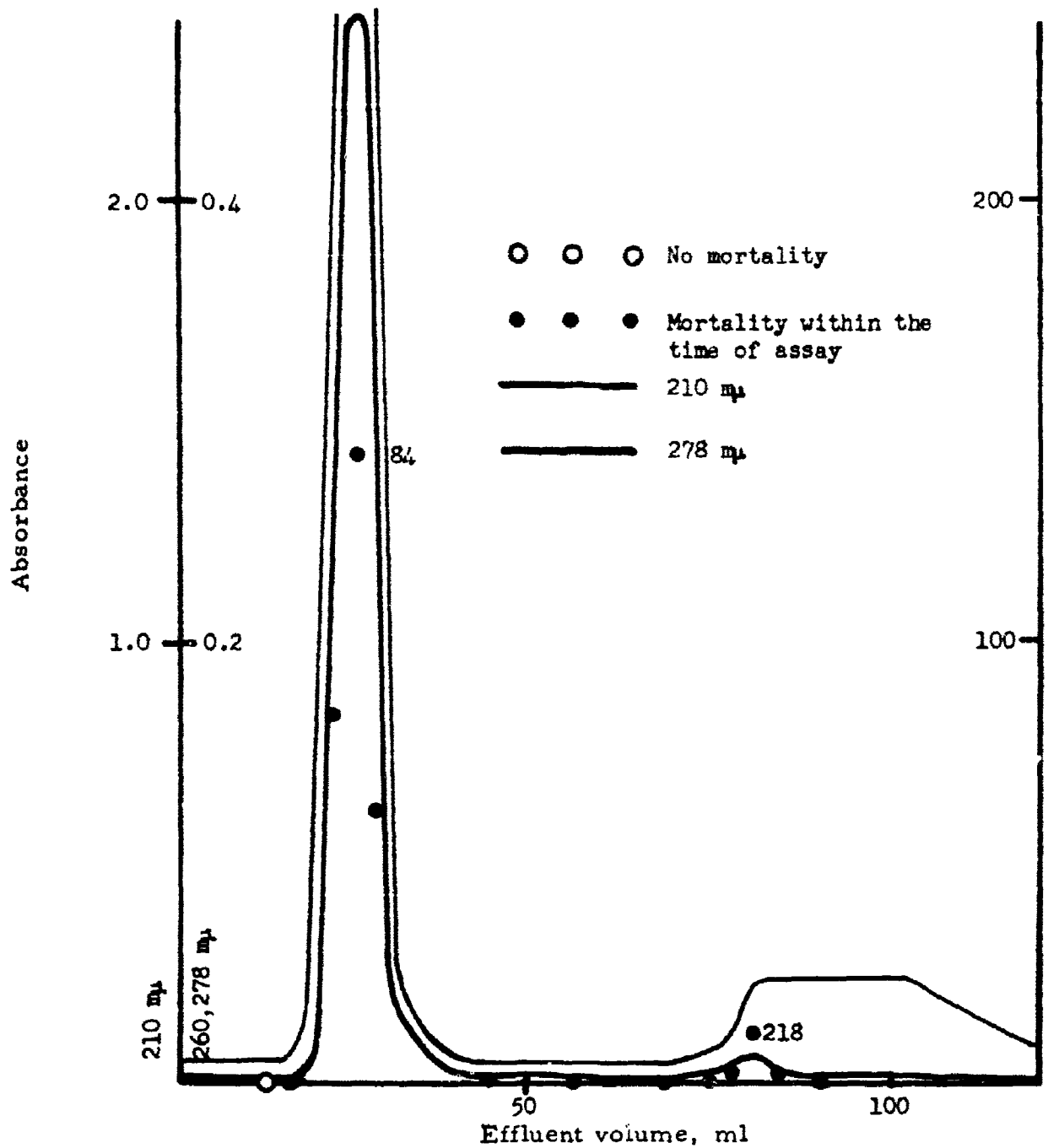
FIGURE 9

SEPHADEX G-75 FRACTIONATION OF BOTULINUM TOXIN TREATED
AT 4°C FOR 7 DAYS WITH SATURATED SALT SOLUTION

[Numerical values at biological-activity points are specific
activities calculated per unit absorption at 278 mμ (value for
untreated toxin = 120)]
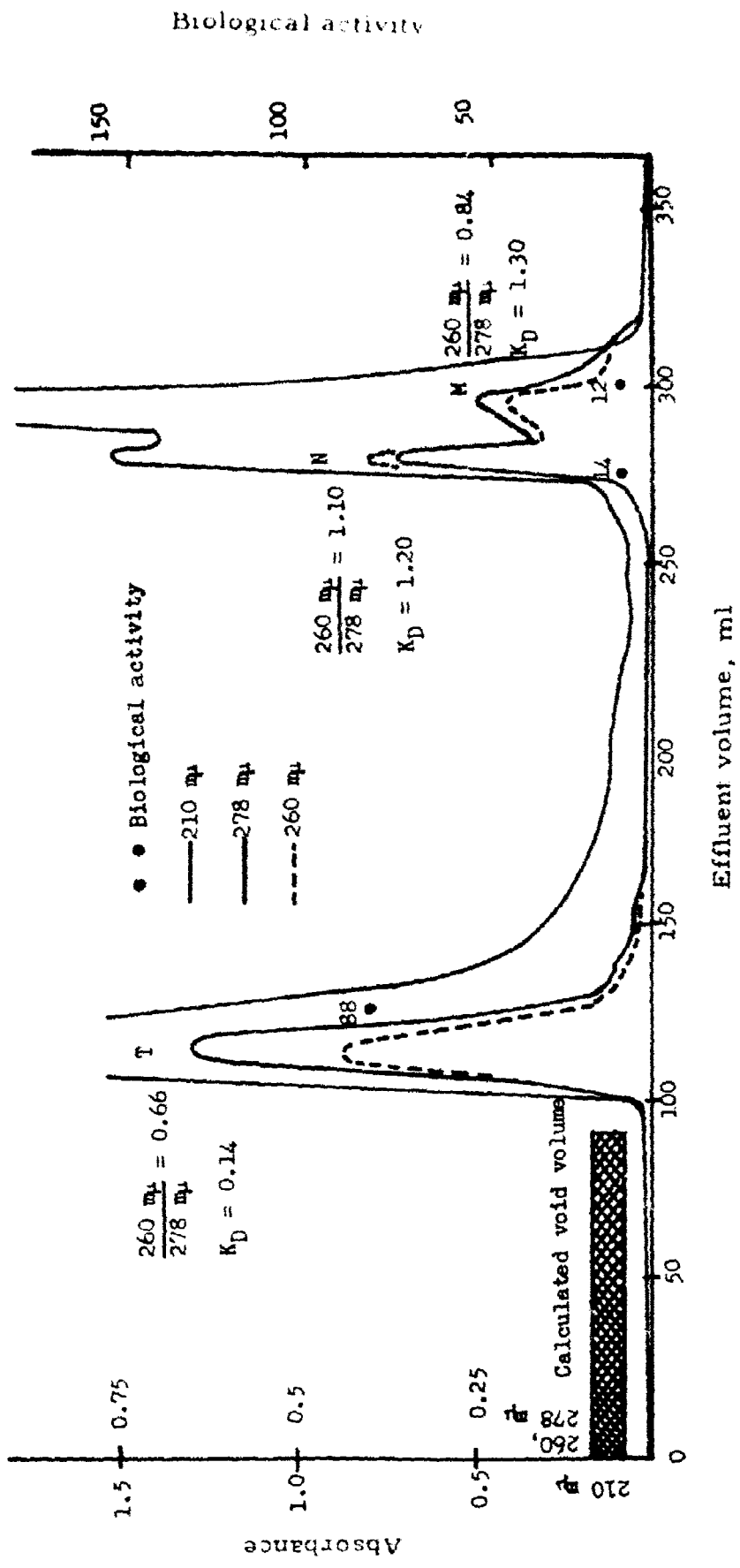
FIGURE 10

SEPHADEX G-75 FRACTIONATION OF BOTULINUM TOXIN TREATED
WITH 0.1 N HCl At 4°C FOR 7 DAYS

[Absorption ratios (260:278 mμ)and calculated distribution coefficient for each peak
are shown; numerical values above biological-activity points are specific activities
calculated per unit absorption at 278 mμ (value for untreated toxin = 120)]

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The effect of time of treatment upon the gel-filtration pattern is shown in figure 11. With increasing time of treatment, the T peak decreases while the M peak increases in size. Areas in the T and M peaks are not directly comparable, since the ordinate is 278 mμ absorbance, not nitrogen content.

The effects of time and temperature are tabulated in table 8. The approximate times for 50% retardation are: $4^{\circ}C$, 8 wk; $25^{\circ}C$, 6 hr; and $37^{\circ}C$, 5 min.

Considerable denaturation was found in the retarded peak at $37^{\circ}C$ in all the time periods and at the lower temperature after prolonged time. Figure 12 shows that the overall process is not first-order. The high temperature dependence is shown in figure 13. The ultraviolet (UV) absorption spectra of the original toxin, the type T peak, and the type M peak are shown in figure 14. The effects of the ionic species, the strength, and the pH of the gel eluant on the yield of the type M peak are shown in table 9. Neutral phosphate at 0.05 M and 0.2μ was optimal as the eluant.

Several large-scale experiments were performed to accumulate type M material for physicochemical and biological characterization. Table 10 summarizes the properties of the type M material in the best preparation. The yield of soluble, undenatured, type M material was 1.3% of the original toxin with 41% toxicity, according to mouse ip LD50 assay on a nitrogen basis. Ultracentrifugation studies showed that sedimentation coefficients (S_{ob}) of the type T material were similar to those of the original toxin $(S_{ob} = 16)$, whereas those of type M were reduced $(S_{ob} = 10.5)$. Differential ultracentrifugation studies showed that most (over 90%) of the original toxin and the type T material sedimented at 144,000 g's in 6 hr, whereas most of type M did not.

We believe we have produced, in low yield, material that is probably smaller in molecular size but that retains considerable toxicity. The approach is an unusual one, consisting of pretreatment with cold mineral acid and subsequent neutralization in dextran gel, followed by elution from the gel. Salt interaction during initial zone formation is probably involved in the dissociation process. The process can be visualized as shown in figure 15. Yields are low, considerable denaturation accompanies the dissociation, and reproducibility is variable.

Sample applied to column

Toxin in 0.1 N HCl acid after 24 hr

Toxin in 0.1 N HCl acid after 6 hr

Toxin in 0.1 N HCl acid after 1.5 hr

Toxin in 0.1 N HCl acid after 22.5 min

Toxin in 0.1 N HCl acid at zero time (zero-time control)

0.1 N HCl acid only (acid control)

Toxin in phosphate buffer at pH 6.8 (phosphate control)

Type M

Type T

pH 5.9

10   20   30   40   50   60

Effluent volume, ml
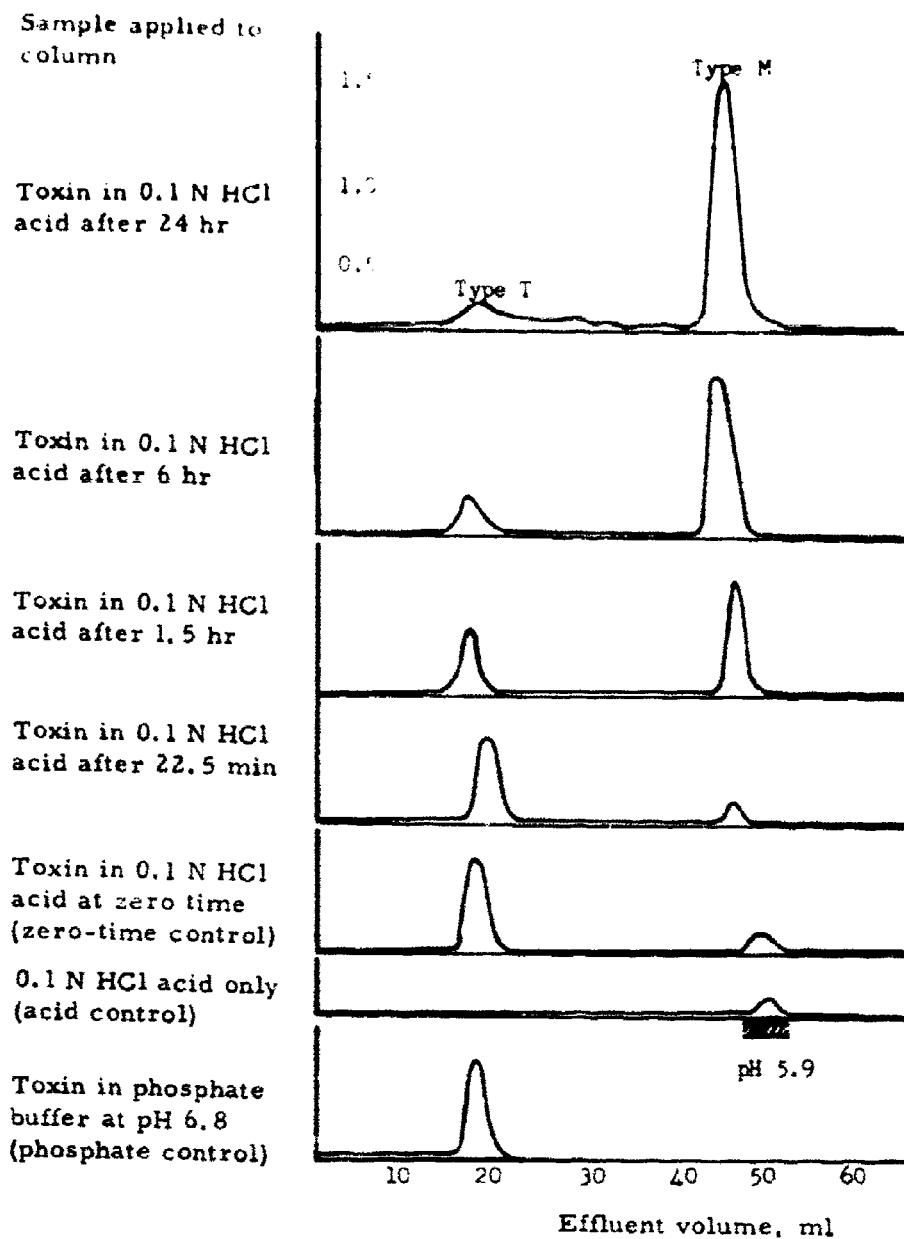
FIGURE 11

ELUTION CURVES OF BOTULINUM TOXIN TREATED FOR INCREASING
LENGTHS OF TIME WITH 0.1 N HCl AT 25°C AND SUBSEQUENTLY
FRACTIONATED WITH SEPHADEX G-75
USING PHOSPHATE ELUANT

(Lowest three curves are control experiments consisting of untreated toxin fractionated with phosphate eluant, 0.1 N HCl eluant only, and initial 0.1 N acid-toxin reaction mixture eluted with phosphate. Similar sample size, column size, and flow rates were used in all experiments. Each experiment is plotted with uniform coordinates)

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TABLE 8

EFFECTS OF TIME AND TEMPERATURE OF TREATMENT OF BOTULINUM TOXIN WITH 0.1 N HCl ON YIELD AND BIOLOGICAL ACTIVITY OF TYPES T AND M MATERIAL ELUTED FROM SEPHADEX G-75 BY PHOSPHATE

Time	4°C Dissociation a/ %	4°C Biological activity b/ T	4°C Biological activity b/ M	25°C Dissociation %	25°C Biological activity T	25°C Biological activity M	37°C Dissociation %	37°C Biological activity T	37°C Biological activity M
0 min				1.5 c/	—	2.2			
5.5 min							52	1.97	0.26
22.5 min				1.5 c/	—	3.8	74	2.2	0.38
1.5 hr				20	1.04	0.31	93	0.20	0.11
6 hr				48	—	0.46			
20.5 hr	0			67	2.8	0.45			
24 hr	8			72	0.53	0.38			
7 days	12								
8 wk	56	—	0.15						

a/ Percent disappearance of peak T in effluent from Sephadex G-75 columns based on total absorbance at 278 mμ. The absorptivity of type T material was linear with protein concentration within the range measured.

b/ LD50 values (LD50 × 10^6/mg of nitrogen) were calculated from 24-hr mortalities after iv injection in phosphate diluent.

c/ Percent appearance of nitrogen in peak M. Absorbance of type M was variable; i.e., two to three times that of type T per unit of nitrogen.

FIGURE 12

RELATIONSHIP BETWEEN TIME OF ACID TREATMENT AT 25°C AND
LOGARITHM OF DEGREES OF CONVERSION TO TYPE M
MATERIAL DURING PHOSPHATE ELUTION
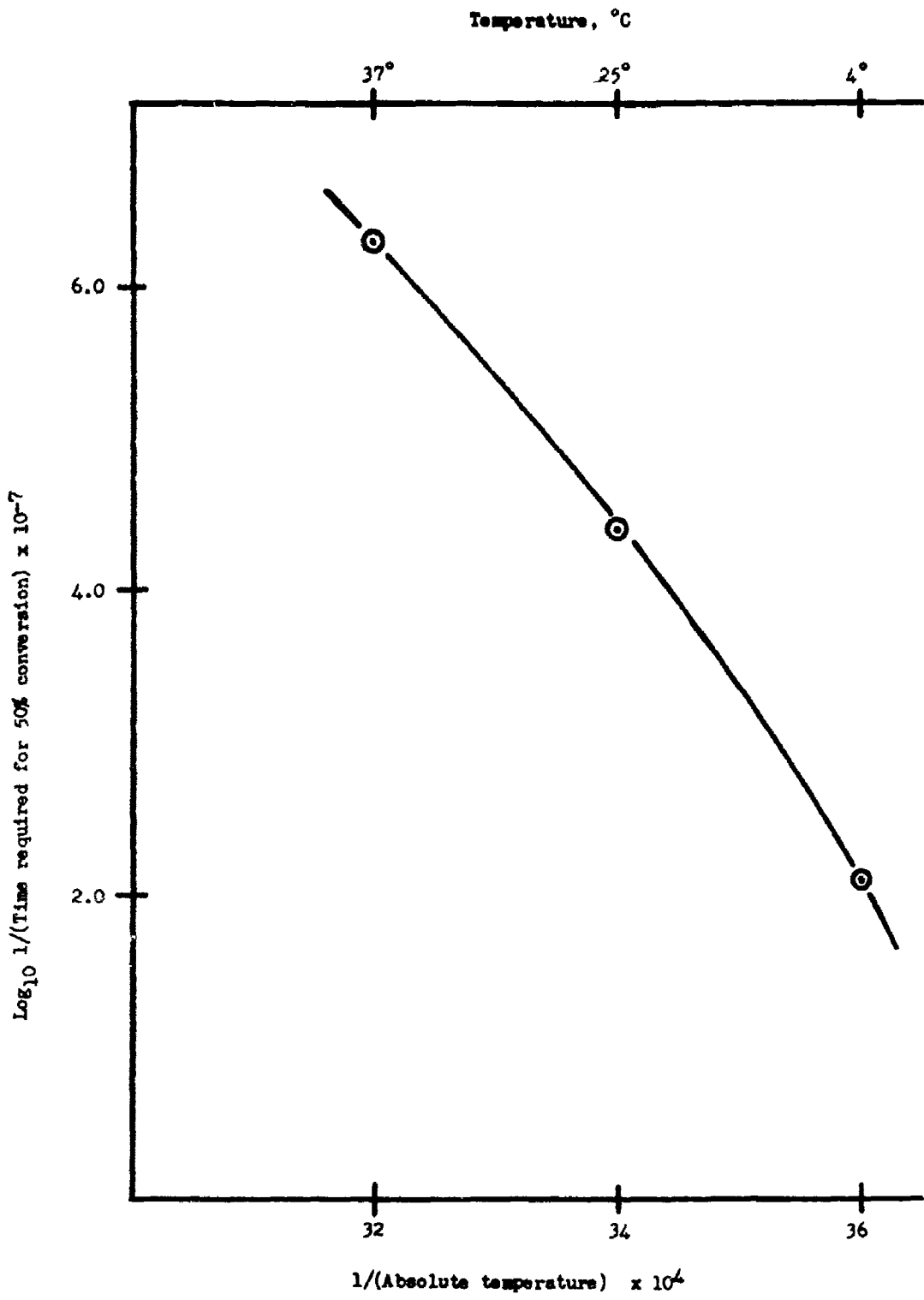
FIGURE 13

RELATIONSHIP BETWEEN RECIPROCAL OF ABSOLUTE TEMPERATURE
DURING ACID TREATMENT AT 25°C AND LOGARITHM OF RECIPROCAL
OF TIME REQUIRED FOR 50% CONVERSION
TO TYPE M MATERIAL

68
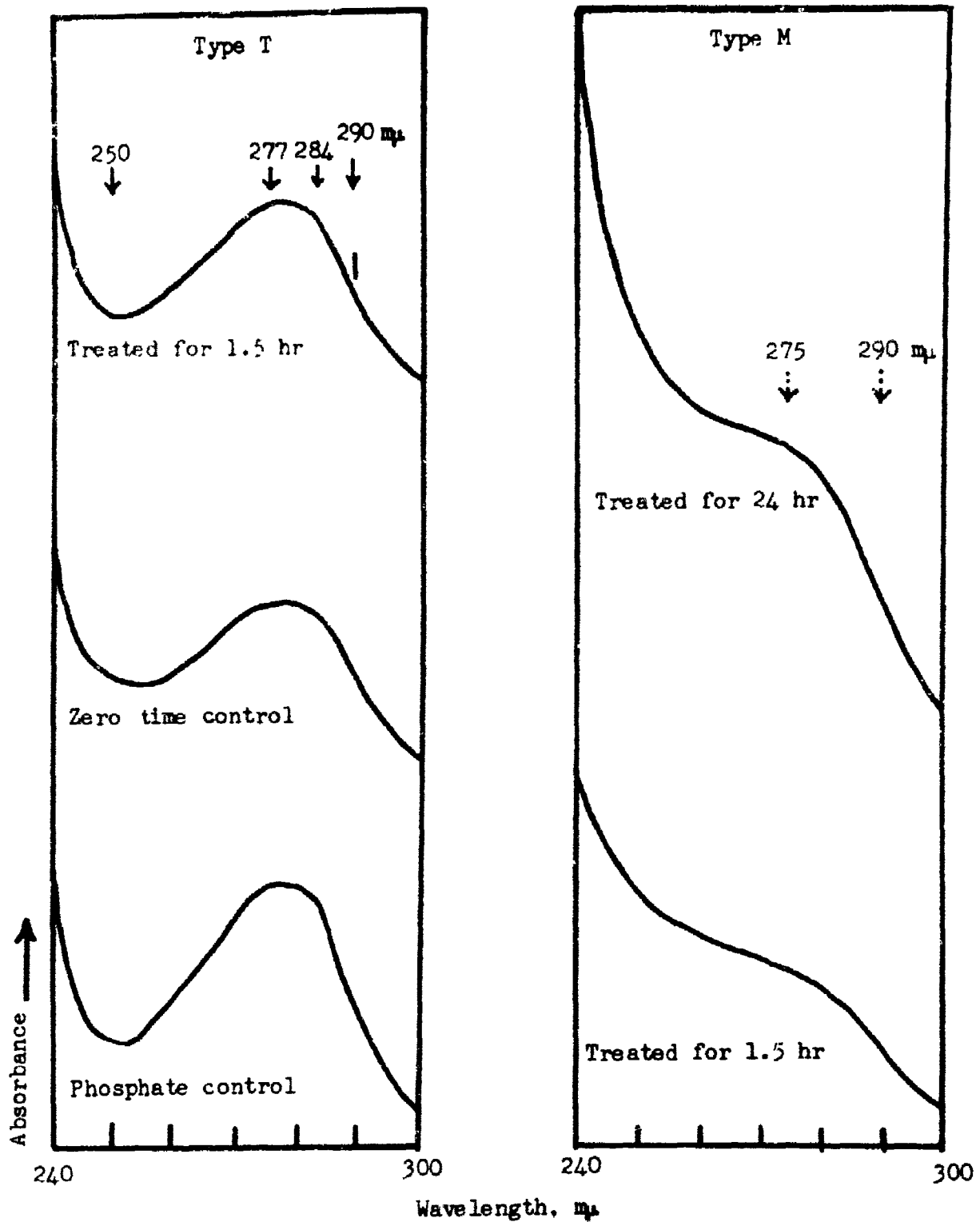
FIGURE 14

UV ABSORPTION SPECTRA OF SEVERAL TYPE T AND M FRACTIONS
OBTAINED IN HCl - PHOSPHATE ELUTION PROCEDURE AFTER ACID
TREATMENT AT 25°C FOR VARIOUS LENGTHS OF TIME

TABLE 9

EFFECT OF IONIC SPECIES, IONIC STRENGTH, AND pH OF ELUANT ON YIELD OF TYPES T AND M MATERIAL ELUTED FROM SEPHADEX G-75

Eluant	pH	Ionic strength	Sephadex	Applied nitrogen recovered %			Comments
				Peak T	Peak M	Total	
H₂O	-	-	G-75	61	0 - 5	66	Small peak at 1.8 void volumes; 260:278 mμ = 0.74
HCl	1.2	0.1	G-75	80	0 - 5	85	Good overall recovery; usual chromatographic procedure
HCl	2.1	0.01	G-75	67	0 - 5	67	—
Acetate	3.8	0.05	G-25	35	0 - 5	38	Low overall yield
Phosphate	6.8	0.02	G-75	82	8	90	Good overall recovery
Phosphate	6.8	0.2	G-75	51	19	70	Typical experiment
Phosphate-NaCl	6.8	0.7	G-75	55	8	63	—

Note: Toxin was treated with 0.1 N HCl at 25°C for 4 hr in all experiments except those using HCl, which were treated similarly but for 1.5 hr. All columns were packed in eluting solvent at room temperature. Sample was applied and eluted from columns at 4°C. Void volume of peak T was typically 1.1 to 1.2; peak M, 2.5 to 4.

TABLE 10

CHARACTERISTICS OF BOTULINUM TOXIN FRACTIONATED IN NEUTRAL PHOSPHATE WITH SEPHADEX G-75 AFTER HCl TREATMENT*

Material	Yield of nitrogen		Specific toxicity		
			LD50/mg N	Percent	S_{ob}
	µg	%			
Original toxin	480	100	152×10^6	100	15.4
Type T, soluble**	243	51	134×10^6	88	16.0
Type M, soluble **	6	1.3	63×10^6	41	10.5
Type M, insoluble**	88	18	9×10^6	6	—

* Treatment: 0.⸱ mg/ml of Type A toxin in 0.1 N HCl for 4.25 hr at
25°C. Column elution: from Sephadex G-75 with 0.05 N phosphate
at pH 6.8.

** All the eluted type T material remained soluble. Some eluted
type M material formed insoluble turbidity that was removed by
slow-speed centrifugation.

Chemical methods for rupturing various linkages were investigated next. Conceivably, single types of bond ruptures may not produce separable subunits, since other linkages maintain conformation of the molecule; hence, sequential methods involving selective and limited rupture of different types of linkages were investigated. Guanidine sulfate was used for hydrogen-bond cleavage, β-mercaptoethanol for disulfide cleavage, cyanogen bromide (CNBr) for methionyl peptide linkages, and N-bromosuccimide (NBS) under conditions selective for tyrosyl peptide linkages. They were all used at pH 1.0 to 2.0 to repress ion-impair formation by positive-charge repulsion.

The relationship between the time of reaction with CNBr at pH 1 and toxicity (measured by the time to death of mice after iv injection) is shown in figure 16. As little as 1 molecule of CNBr per methionyl linkage reduced the potency of the toxin within several hours. The presence of few methionyl linkages in the toxin molecule favored production of larger toxin fragments.
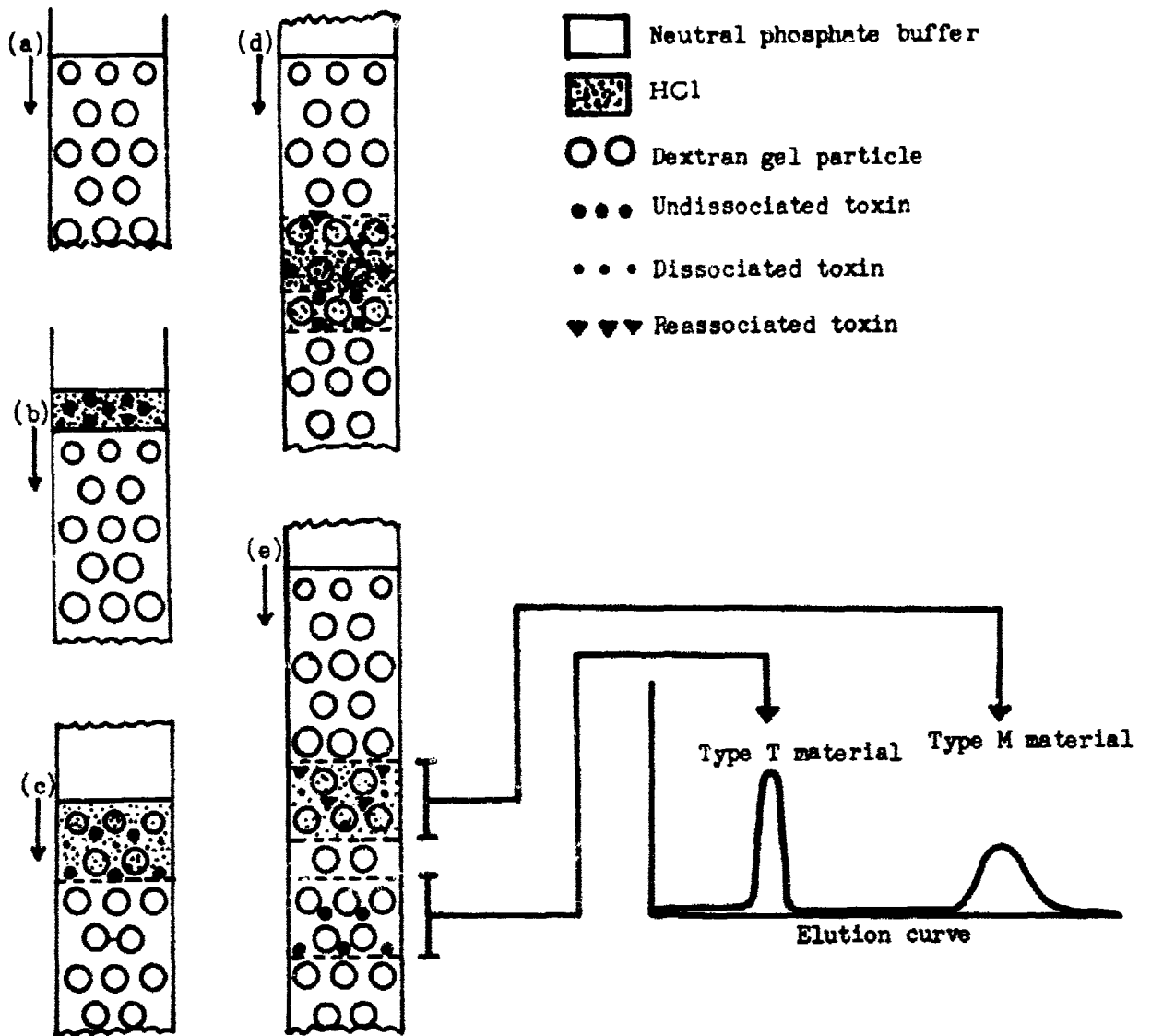
FIGURE 15

POSSIBLE MECHANISM OF HCl - PHOSPHATE DISSOCIATION PROCESS
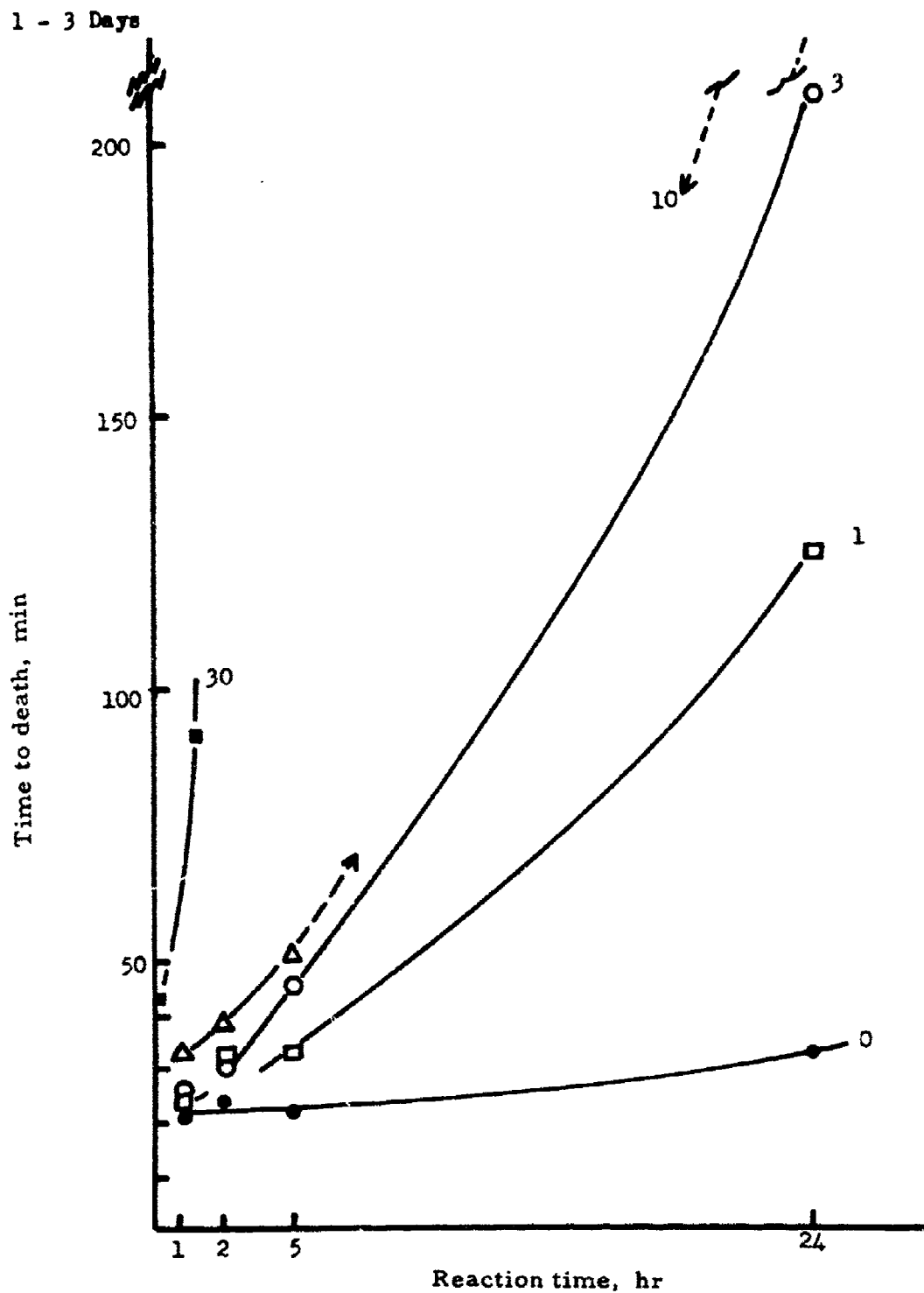
FIGURE 16

EFFECT UPON TOXICITY OF REACTION OF BOTULINUM TOXIN WITH
CNBr FOR VARIOUS TIME PERIODS AND WITH
VARIOUS AMOUNTS OF REAGENT

(Molar ratio of reagent to methionine residues are indicated)

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The toxin was next treated with CNBr at pH 1 for 2.5 hr at 25°C in sulfuric acid in the presence of guanidine sulfate and β-mercaptoethanol. It was dialyzed in a tight casing to remove the reagents and was fractionated in the cold (4°C) with Sephadex G-200 in 0.1 N sulfuric acid at pH 1. The elution curve is shown in figure 17. A small retarded peak with slight biological activity was found.

At a concentration of 3 mg/ml, the toxin was next treated with 3 molecules of CNBr per methionyl linkage in 0.1 N sulfuric acid at pH 1 for 4 hr at 25°C both with and without 1.5 M guanidine sulfate. The schlieren pattern of the reaction mixture at pH 1 taken at 144,000 g's is shown in figure 18. A plot of the sedimentation rates is shown in figure 19. The middle component with S_{ob} = 11.7 (pH 1) sedimented at the same rate as the original toxin. Two sedimented faster and two sedimented slower than the middle one.

We next utilized sequential enzymatic and chemical treatments for toxin fragmentation. Figure 20 shows the schlieren pattern of toxin treated first with pepsin at pH 2.0 for 1 hr at 25°C, followed by 1.5 M guanidine both with and without 0.3 molecule of NBS per methionyl linkage at pH 1.0 for 1 hr at 4°C. Apparently, pepsin and guanidine sequentially produced a small amount of slower-sedimenting component. Inclusion of the NBS with the guanidine increased the size of the slower-sedimenting component.

In view of the oral toxicity of botulinum toxin, limited proteolysis of the toxin by digestive enzymes might yield a toxic fragment. Different enzymes were used in sequence under conditions that limited the rate and extent of proteolysis, in order to retain fragments of sufficient size to have toxicity.

In one series of experiments, toxin was digested with dog-stomach scrapings at pH 2 followed by duodenal scrapings at pH 6.8, with both treatments at 0.2 ionic strength or less. In another series, toxin was digested under similar conditions with pepsin followed by pancreatin. Schlieren patterns of the digestion mixtures are shown in figure 21. Treatment with stomach enzyme produced one rapidly moving peak (S_{ob} = 14.5), whereas stomach enzyme followed by duodenum enzyme produced three slower moving peaks (S_{ob} = 10.1, 8.1, and 3.95). Stomach enzyme followed by duodenum enzyme and pepsin followed by pancreatin apparently produce similar sedimentation patterns, which contain two major fragments.

Solid dots = 278 mμ absorption

Open dots = 260 mμ absorption

M   Nitrogen content 3 x baseline
    slight biological activity

0.1 N sulfuric acid, ml

Absorbance at 278 mμ
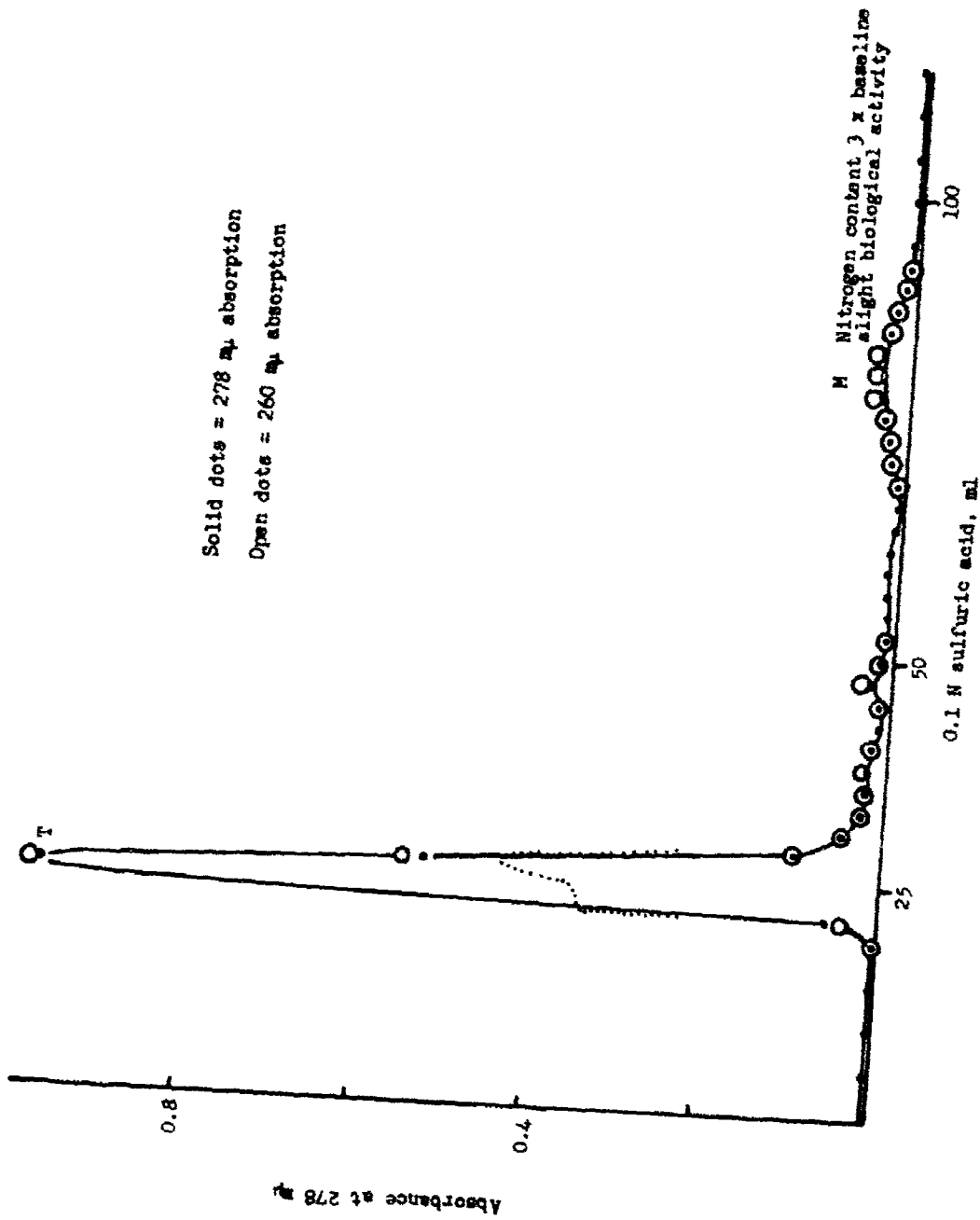
FIGURE 17

EFFLUENT CURVES OF BOTULINUM TOXIN TREATED WITH CNBr, GUANIDINE
SULFATE, AND β-MERCAPTOETHANOL (SOLID LINE) AND WITH
CNBr ONLY (DOTTED LINE)

Expt 236-22

Run 104
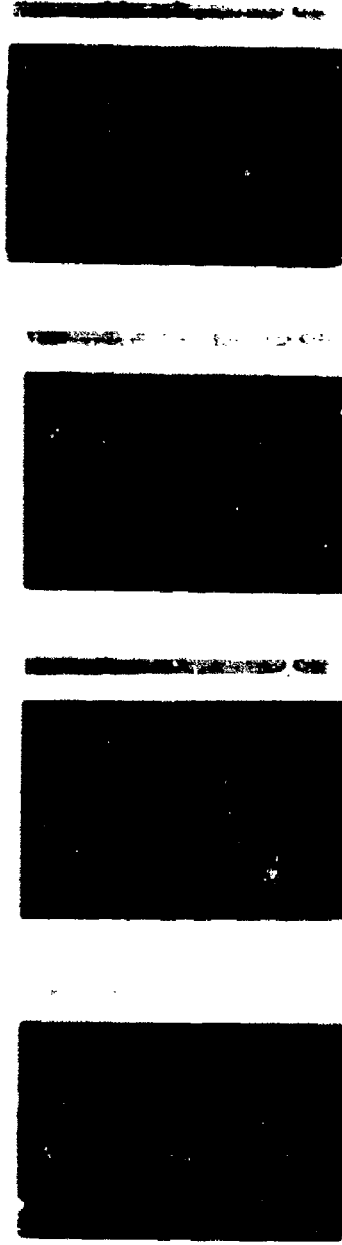
FIGURE 18

SEDIMENTATION-VELOCITY PATTERNS OF BOTULINUM TOXIN AFTER TREATMENT
WITH CNBr AND GUANIDINE IN 0.1 N SULFURIC ACID

(Bottom pattern, with guanidine sulfate; top pattern, without guanidine sulfate;
toxin was centrifuged at 42,040 rpm at 5°C; one 12-mm cell had wedge
window; photographs taken at 16 min for first frame, and thereafter interval
was 32 min)
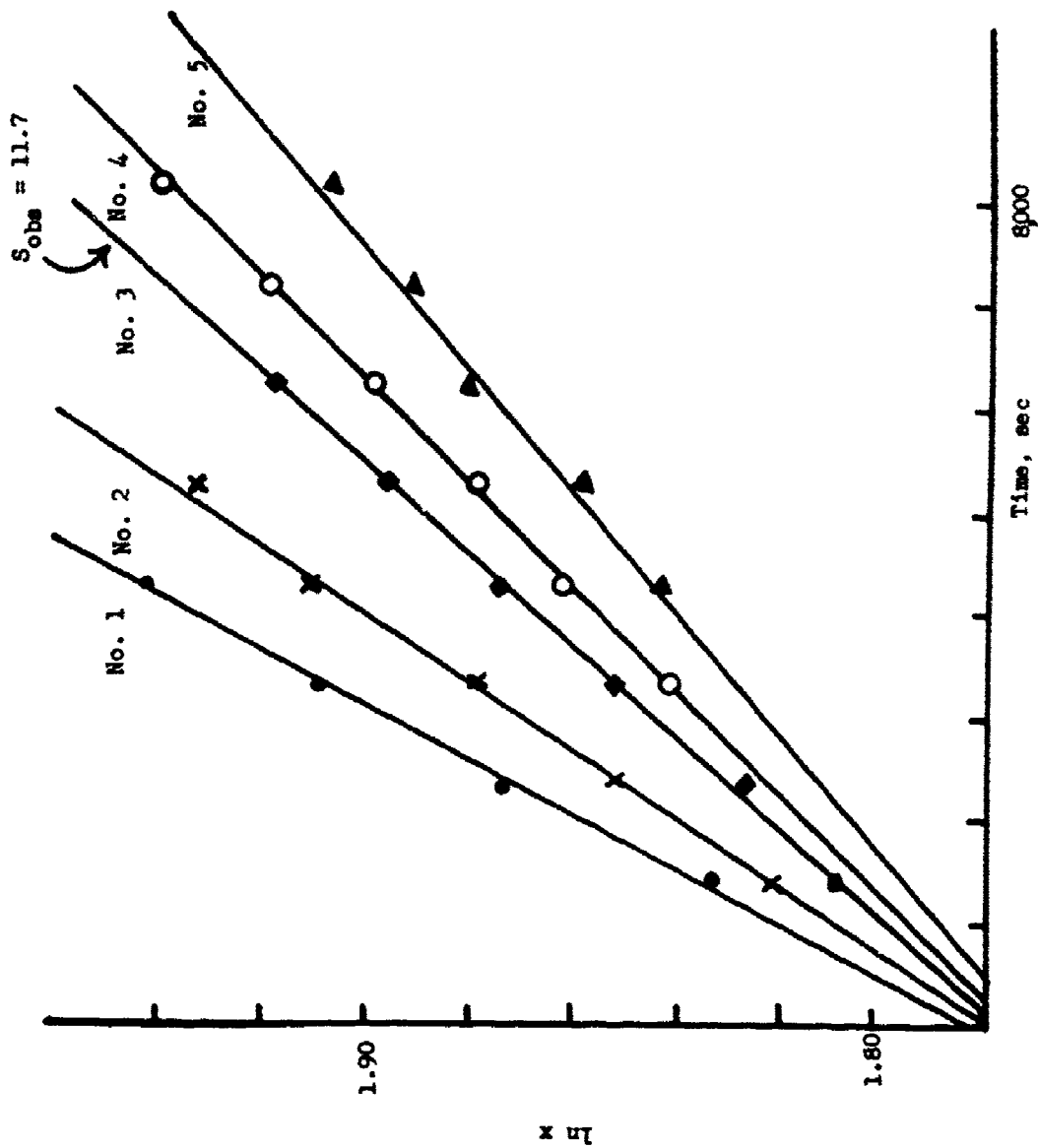
FIGURE 19

SEDIMENTATION RATES OF BOTULINUM TOXIN AFTER TREATMENT WITH CNBr AND
GUANIDINE (LOWER PATTERN OF FIGURE 18)

Expt 775-4

Run 128

FIGURE 20

SEDIMENTATION-VELOCITY PATTERNS OF BOTULINUM TOXIN TREATED
SEQUENTIALLY WITH PEPSIN AND NBS (30-MM CELLS)

(Top pattern, with NBS; bottom pattern, without NBS; photographs taken at
16 min for first frame, and thereafter interval was 32 min; centrifuged at
42,040 rpm and 5°C; wedge window used)
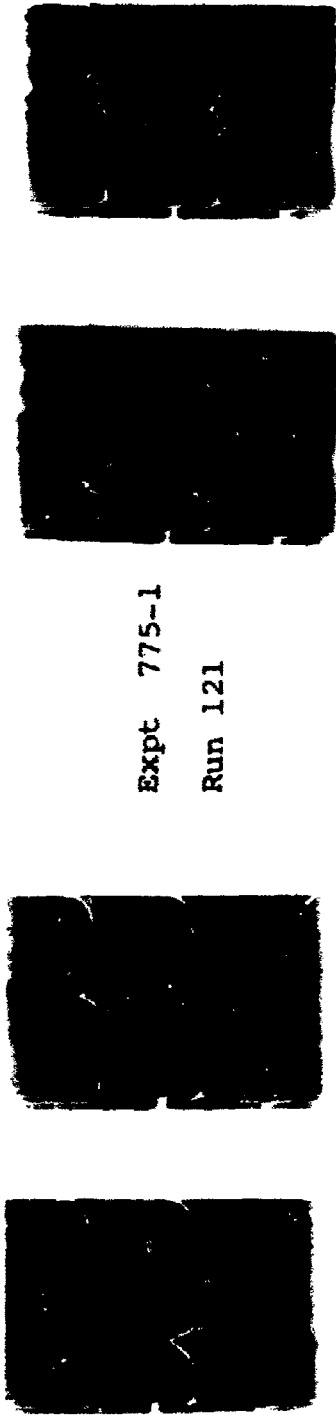
Expt 236-24
Run 113

Expt 775-1
Run 121

FIGURE 21

SEDIMENTATION-VELOCITY PATTERNS OF ENZYMATIC DIGESTS OF
BOTULINUM TOXIN (12-MM CELLS)

(Photographs taken 16 and 48 min after attainment of
59,780 rpm; wedge window used)

Top: stomach pH 2.0; duodenum pH 4.4   Top: pepsin pH 2.0; pancreatin pH 4.4
Bottom: stomach pH 2.0   Bottom: stomach pH 2.0; duodenum pH 4.4

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The ionic environment is apparently critical in these experiments. Although results were somewhat variable, more of the slowest-moving component was obtained when the pH shifted from 4.4 to 6.3 before or after the pancreatic digestion (figure 22).

Thus, pepsin alone produced only the rapidly sedimenting components (S_{ob} = 15 to 19), whereas pepsin and pancreatin or pancreatin alone produced additional peaks ranging from S_{ob} = 1.15 to 4.5, 8.13, and 10.10. The slowest, 1.15, was observed with a synthetic boundary cell.

Sephadex G-75 fractionation in a preparative-scale experiment with 3 mg of toxin is shown in figure 23. The nonretarded peak had a 260:278-mμ absorption ratio similar to that of the original toxin, whereas the retarded peak was similar to that of the type M material previously described. Several experiments with up to 18 mg of toxin were completed. The enzymes generally appeared in the type M moiety, accounting for two-thirds of the absorbance in this peak. The toxicity of the nonretarded material varied in different experiments from 10% to 90% of the original toxin in relation to nitrogen, whereas that of the retarded material was 1% to 40%. The latter material included some nitrogen from enzymes.

The enzyme-digested fractions were subjected to immunophoresis in agar gel. Samples were applied to the center of the gel and were subjected to electrophoresis. Antitoxin, applied in a slit parallel to the toxin electrophoresis path, was then allowed to diffuse against the toxin. Comparable precipitin lines were obtained from the nonretarded peak (T) and the original toxin. No precipitin line, however, was found with the retarded peak (figure 24, peak M).

Other fractionation methods that were investigated included ultracentrifugation at 173,000 'g's, density-gradient centrifugation in sucrose medium, and continuous-flow electrophoresis (figure 25). In figure 25, the toxicity scale is logarithmic, but the absorbance scale is linear. The peaks of interest are those showing considerable toxicity without 278 mμ absorbance, which suggests specific toxicity greater than the original material.

The toxin at pH 6.8 was labeled by the triiodide procedure with iodine-125. The list that follows summarizes some of the properties of the toxin at low and high levels of triiodide. At 100 equivalents, we obtained a good yield of labeled toxin, a low percentage of label on the protein (obtained by calculation, not by direct analysis of iodine), and high toxicity. At a high level of labeling (1,000 equivalents), practically no toxicity was obtained. Hence, 100 to 1,000 equivalents of iodide per mole of toxin is a critical interval.

Expt 775-13

Run 162
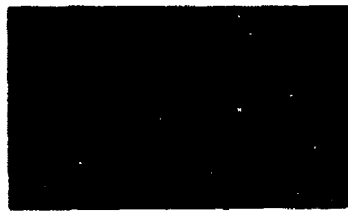
Expt 775-12

Run 160
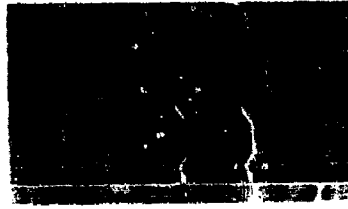
FIGURE 22

SEDIMENTATION-VELOCITY PATTERNS OF ENZYMATIC DIGESTS OF BOTULINUM TOXIN (30-MM CELLS)

(Photographs taken 16 and 32 min after attainment of 50,740 rpm; wedge window used)

Top: without toxin; pepsin pH 2.0; pancreatin pH 4.4, then adjusted to pH 6.3

Top: pancreatin pH 4.4, then adjusted to pH 6.3

Bottom: same with toxin

Bottom: pepsin pH 2.0; pancreatin pH 4.4, then adjusted to pH 6.3

Column size: 25 ml

Amount of
treated toxin: 3 mg

Effluent: pH 6.3

● 278 mμ

✗ 260 mμ

Effluent
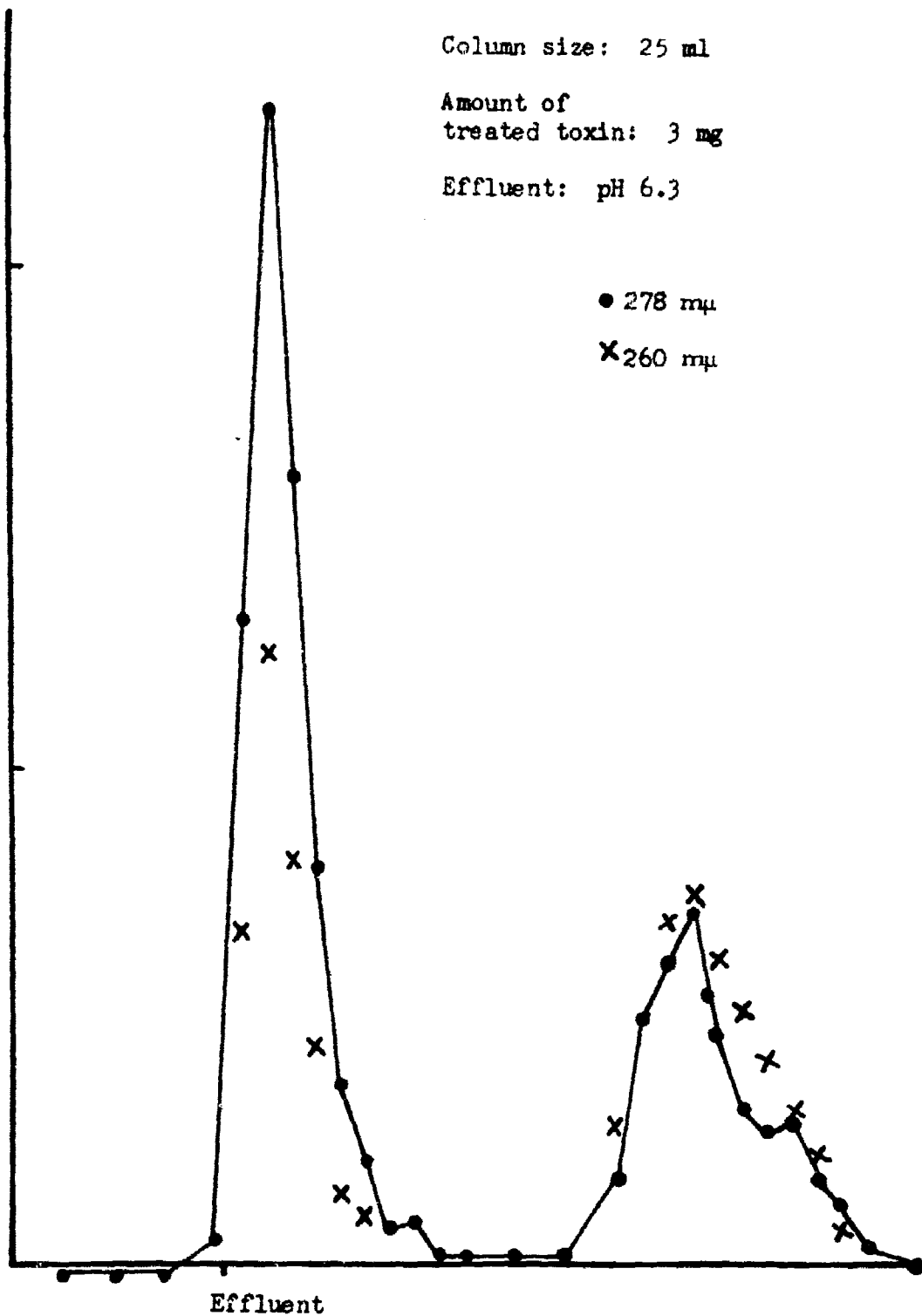
FIGURE 23

SEPHADEX G-75 FRACTIONATION OF TOXIN TREATED
SEQUENTIALLY WITH PEPSIN AND PANCREATIN

(Column size, 25 ml; amount of treated toxin, 3 mg)

82
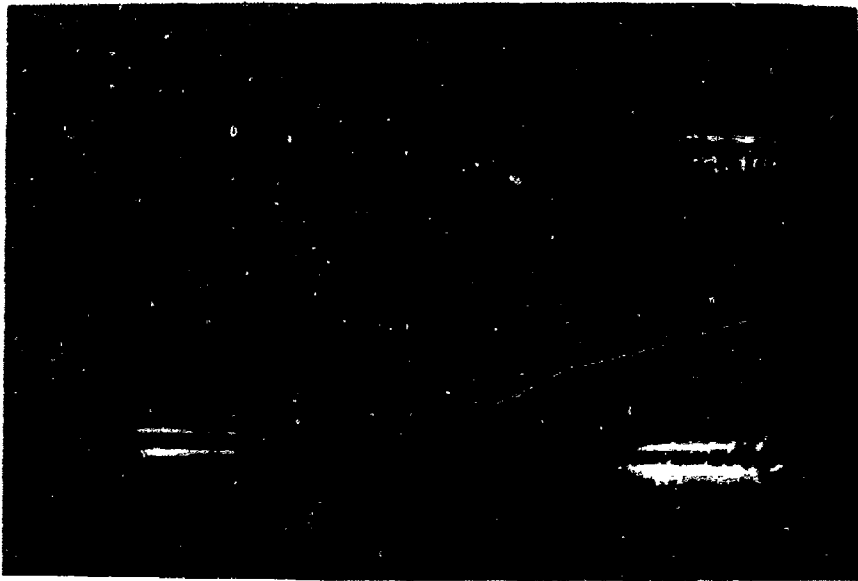
Nonretarded peak T

Original toxin

Nonretarded peak T

Retarded peak M

FIGURE 24

IMMUNOPHORESIS OF SEPHADEX G-75 FRACTIONS FROM
PEPSIN-PANCREATIN-TREATED TOXIN
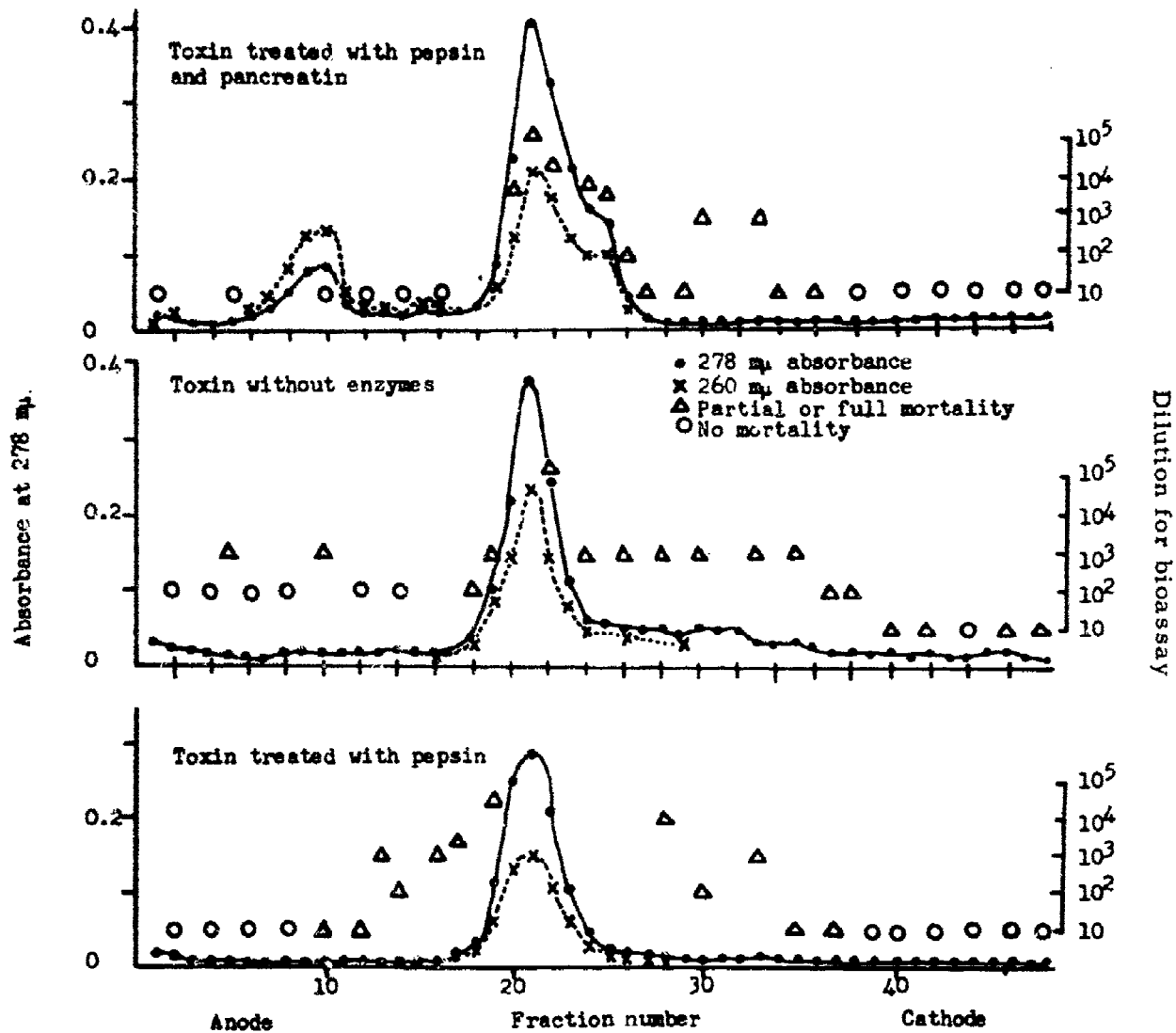
FIGURE 25

CONTINUOUS-FLOW ELECTROPHORETIC FRACTIONATION OF BOTULINUM
TOXIN TREATED SEQUENTIALLY WITH ENZYMES

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	Low level	High level
Equivalents of triiodide per mole of toxin	100	1,000
Percent yield of labeled toxin	90	50
Percentage of label on protein	0.33	3.6
Calculated average equivalents of diiodotyrosine formed per mole of toxin	0.17	9
Relative toxicity (uniodinated toxin = 100)	24.3	0.002

The data listed below show the effects of intermediate levels of labeling. Apparently, 100 equivalents produce maximal labeling with maximal toxicity. As toxicity is lost, hemagglutinating activity is lost. Figure 26 shows sedimentation patterns of the uniodinated species versus the iodinated species at two levels of iodination. The low level of labeling (100 equivalents) produced no change in the S_{ob}. The high level (1,000 equivalents) produced not only less material (because of denaturation due to manipulation and insolubilization of much of the toxin), but also a species that sedimented slower.

	Intermediate levels			
Equivalents of tri-iodide per mole of toxin	100	200	400	800
Degree of labeling (counts/min/μg N)	2,980	4,350	9,680	13,300
Relative toxicity (uniodinated toxin = 100)	44	32	4.5	0.16
Hemagglutinating activity	Normal	Normal	Abnormal	Abnormal

Table 11 summarizes the sedimentation behavior at various levels of labeling.

85

A. Uniodinated
   $S_{obs} = 17.4$

B. 1000 X
   $S_{obs} = 12.9$

C. Uniodinated
   $S_{obs} = 16.6$

D. 100 X
   $S_{obs} = 16.6$
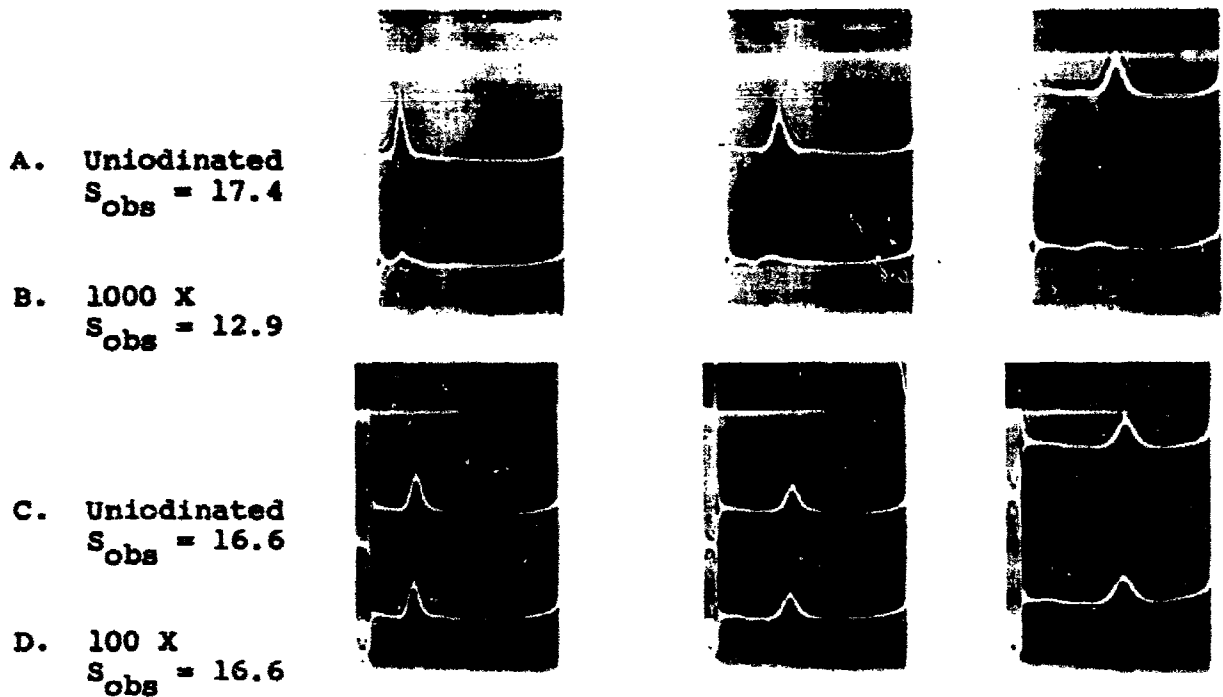
FIGURE 26

SEDIMENTATION DIAGRAMS OF UNIODINATED BOTULINUM TOXIN
COMPARED WITH TOXIN IODINATED AT 100 OR
1,000 EQUIVALENTS PER MOLE

(Speed, 42,040 rpm; temperature, $5.0^{\circ}C$; time interval between
pictures, 16 min; buffer, 0.05 M phosphate, pH 6.8)

TABLE 11

OBSERVED S_{ob} OF BOTULINUM TOXIN
IODINATED AT VARIOUS LEVELS*

Equiv iodine/ mole toxin	$S_{ob} \times 10^{-13}$	
	Expt 1	Expt 2
None	17.4, 16.6	—
100	16.6	17.2
200	—	17.2
400	—	17.0
800	—	17.2
1,000	12.9	—

* Same experimental conditions were used for each centrifugation.

The toxin was next labeled with iodine-125 using 50 equivalents of triiodide per mole of toxin. The iodinated toxin had the same toxicity and S_{ob} as native toxin. Labeled toxin that was diluted with unlabeled carrier toxin was treated sequentially with pepsin and pancreatin and fractionated with Sephadex G-75. The results showed up to six times the amount of label in the retarded moiety compared with the untreated peak. Other experiments showed that the unlabeled toxin was cleaved almost as readily as the labeled toxin. It was tentatively concluded that the cleavage was unsymmetrical, with a concentration of iodine in the cleaved component.

The iodine-labeling technique is believed to be useful in studying mechanisms of cleavage in terms of molecular geometry. For example, alternative mechanisms, such as those shown in figure 27, can be distinguished. It would be important to establish the proportion of toxin molecules that are iodinated, however, before conclusions could be drawn.
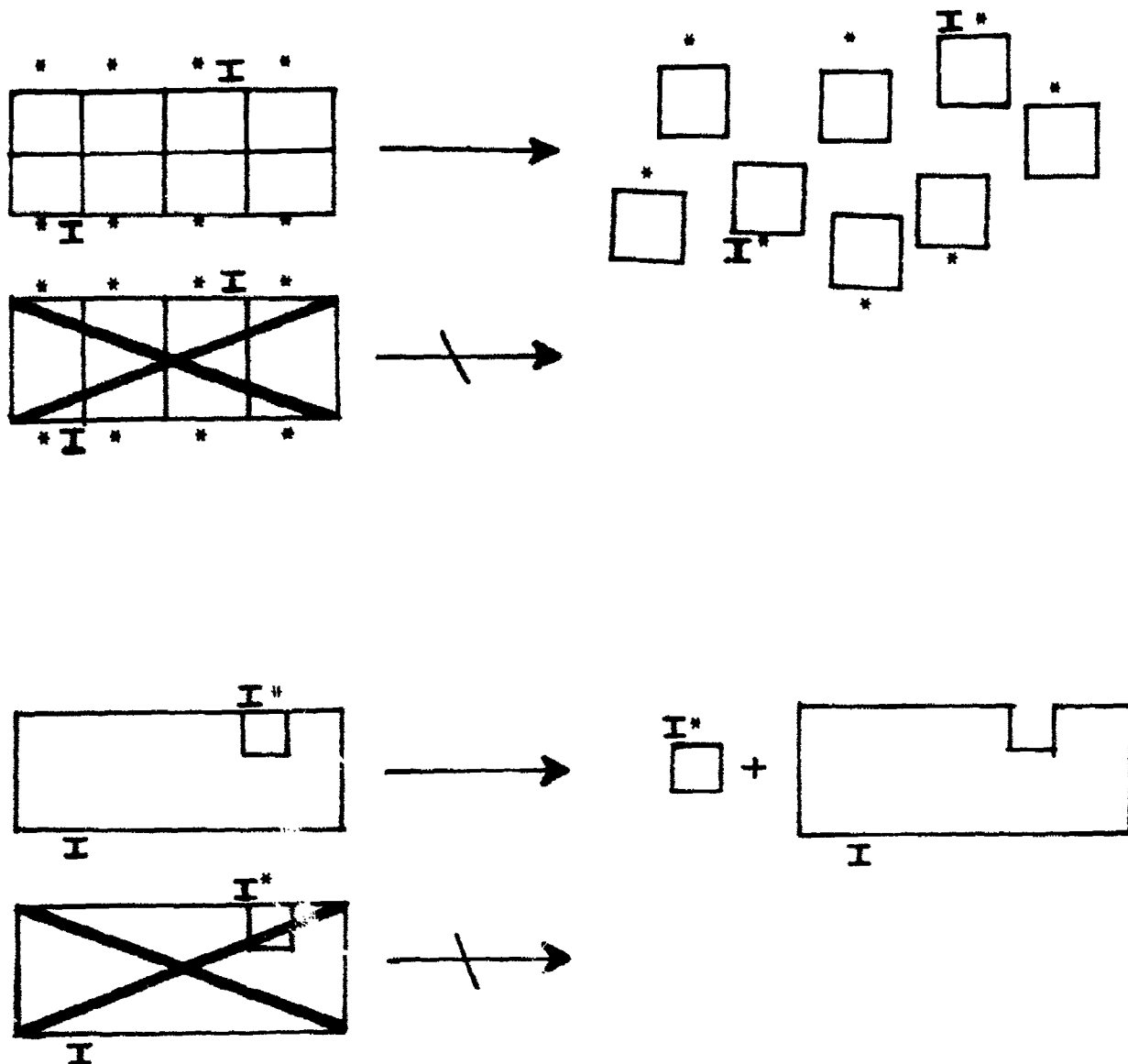
I = Radioactive iodine label

* = Neurotoxic site

FIGURE 27

ALTERNATE MECHANISMS OF ENZYMATIC CLEAVAGE OF LABELED
BOTULINUM TOXIN MOLECULE

DISCUSSION

Dr. Sowinski (Albany Medical College): In iodination, you would expect the tyrosine residue to be affected, would you not? There is some work in the literature by Dr. Boroff showing that photooxidation of botulinum toxin destroys the tryptophan residue with simultaneous loss of toxicity. Could you say that, when you iodinate all of the tyrosine, the tryptophan would be affected?

Dr. Riesen: The findings of Dr. Boroff are very interesting. There are various ways to destroy the activity of botulinum toxin. To necessarily associate the active toxic site with a particular site affected by the method of treatment might be hazardous. One can, for example, visualize the action of iodine under drastic conditions as simply unfolding the molecule and exposing additional surface.

Under these conditions, one can enforce additional labeling, even with triiodide. One can then oxidize tryptophan and, perhaps, other amino acids, so that the loss in activity cannot necessarily be associated with tyrosine.

Question: What was the pH?

Dr. Riesen: 6.8.

Dr. Sternberger (CRDL): I think that at pH 6.8 there is too little oxidation for it to be an important factor. At pH 5.0, of course, you do get oxidation. At pH 8.0, there is none.

Dr. Riesen: Yes. Dr. King used iodinated toxin in his work, and I believe he used a slightly higher pH.

Dr. King (IITRI): It was 7.0 to 7.5.

Dr. Sternberger: Could not oxidation be excluded if you worked at pH 8.0, so that you are sure that tyrosine or histidine are iodinated?

Dr. Riesen: To determine this, one would have to analyze the iodinated species.

Dr. Sternberger: I think that has been rather rigorously analyzed in other proteins.

Dr. Riesen: Yes.

Dr. Sternberger: And those conditions have been obtained.

Dr. Riesen: Pepsin and other biologically active proteins can generally be labeled to the extent of only 1 equivalent of iodine per mole of protein. Beyond that, you begin to lose activity. The evidence suggests that our iodination procedures iodinate relatively few toxin molecules, and then only 1 mole per toxin. Hence, we cannot be sure that the retained toxicity of the lightly iodinated species is that of iodinated molecules.

Dr. Sternberger: It is very possible that you have a critical tyrosine group on the molecule and that this is being destroyed. Would you think that, for instance C^{14} acetylation may be less destructive of specific activity of the molecule?

Dr. Riesen: There are a lot of tyrosines on the molecule. Furthermore, if the site of action is on a particular subunit, the attempt to find a particular tyrosine might be difficult.

Dr. Sternberger: In the active material, I think you did have only 0.17 equivalent of iodine per protein molecule, which would mean you have a lot of material that does not contain any iodine at all.

Dr. Riesen: That is right.

Dr. Sternberger: And this would not in itself indicate a very strong effect of iodine, especially on tyrosine, at pH 8.

Dr. Riesen: Perhaps.

Dr. Sternberger: When you treat the molecule with CNBr in the presence of guanidine hydrochloride and then separate it in HCl, do you get reassociation in your original conformation if you take the separated molecules and remove the agent?

Dr. Riesen: There is a certain amount of insoluble material obtained in such a reaction. I am not sure, with our data, that we can distinguish between reassociation at the end of the reaction and denaturation due to the process itself.

Dr. Sternberger: Could you conceivably separate in propionic acid instead of hydrochloride, under milder conditions than using CNBr?

Dr. Riesen: Possibly. We did not try weaker acids.

Dr. Sternberger: I wonder whether the peaks you have that are not quite separated can actually be separated by recycling chromatographically? Could you separate them for preparative purposes, or would they denature too much if you recycle again by using two columns and siphoning off one peak as it separates?

Dr. Riesen: I do not think this would change the basic process occurring in the column. Greater efficiency, however, might be achieved. Whether denaturation would be cumulative is difficult to answer. These experiments that I have described were done some time ago, and I do believe that there are better approaches at the present time.

Dr. Sternberger: Techniques develop so fast and the work always takes time; it is slower than the development of new techniques. This work is very beautiful and important.

Dr. Riesen: Evidence obtained by several investigators suggests that milder approaches than HCl treatment, oxidative degradation, or proteolytic enzymes will dissociate Type A botulinum toxin. It would appear that drastic degradative procedures are best applied to the characterization of the dissociated subunit.

LETHAL SUBUNITS OF TYPE A <u>CLOSTRIDIUM BOTULINUM</u> TOXIN

Dr. Maurice E. King
Illinois Institute of Technology Research Institute

This paper summarizes some of the results obtained in a program sponsored by the Chemistry Division of CRDL that was monitored by Mr. Samuel Sass. The program was initiated to develop a better assay method for botulinum toxin; however, early in the program, some results indicated a possible fragmentation of the large, crystalline, toxic units into smaller toxic subunits. Consequently, attempts were made to isolate and characterize these subunits.

The initial evidence for fragmentation was obtained during immunophoresis studies. This procedure involves electrophoresis of the crystalline toxin in an agar film and subsequent diffusion of both the toxin and its antibody to form a precipitin reaction. Figure 28 illustrates four types of reactions. One set of precipitin lines is seen for fresh crystalline toxin. Additional lines are present: for "mud" (the acid precipitate, figure 28), for toxin that had been stored in a refrigerator for about a year, and for iodinated toxin.

In order to relate toxic activity to the position of the precipitin lines, crystalline toxin and "mud" were separated by electrophoresis without allowing a reaction with the antibody. The agar was divided into sections, homogenized in gel phosphate, and injected into mice. Figure 29 and table 12 show that toxicity was found in sections of the agar in which no antigenic material had previously been found. Thus, toxin fragments with different electrophoretic mobilities and different antigenic properties were apparently produced.

TABLE 12

MORTALITY OF MICE INJECTED WITH AGAR SECTIONS*

Dilution	Mortality fraction				
	Section				
	1	2	3	4	5
1:5	0/4	4/4	4/4	4/4	4/4
1:10	0/4	4/4	4/4	4/4	4/4
1:100	0/4	3/4	4/4	4/4	3/4
1:400	0/4	2/4	4/4	4/4	3/4
1:2,000	0/4	0/4	4/4	4/4	3/4

* From 6-μg crystalline-toxin suspension subjected to electrophoresis.

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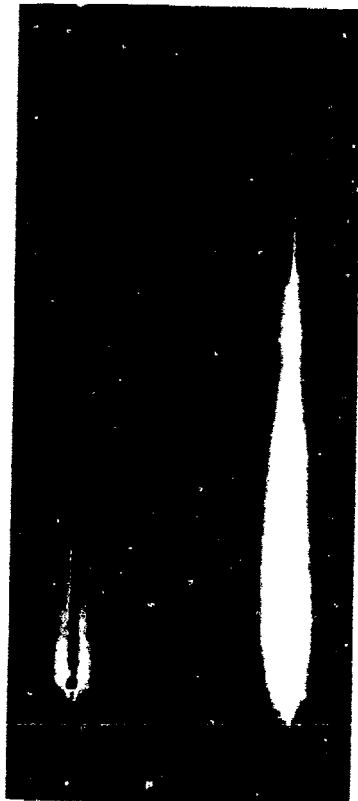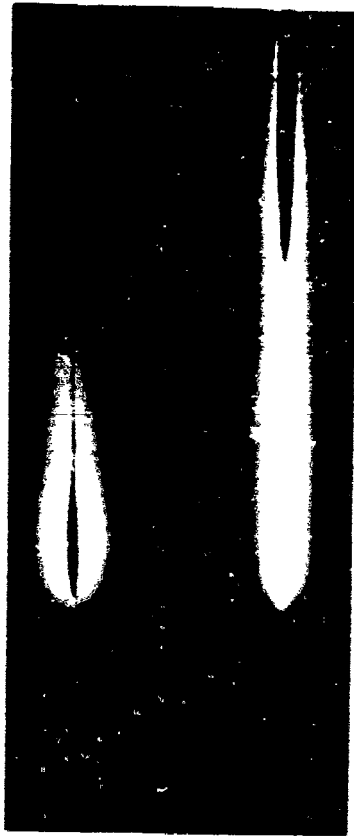
Second Acid Precipitate
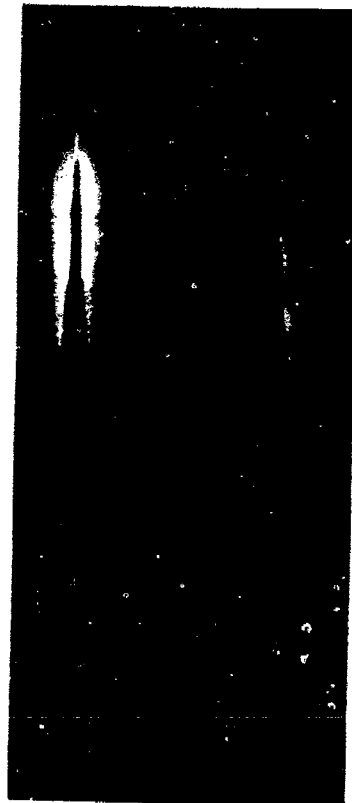
Iodinated Toxin

Crystalline Toxin

Stored Toxin

FIGURE 28

IMMUNOPHORESIS OF TOXIN SOLUTIONS

94

Precipitin lines from
crystalline toxin

Origin

Cathode

Anode

1 2 3 4 5
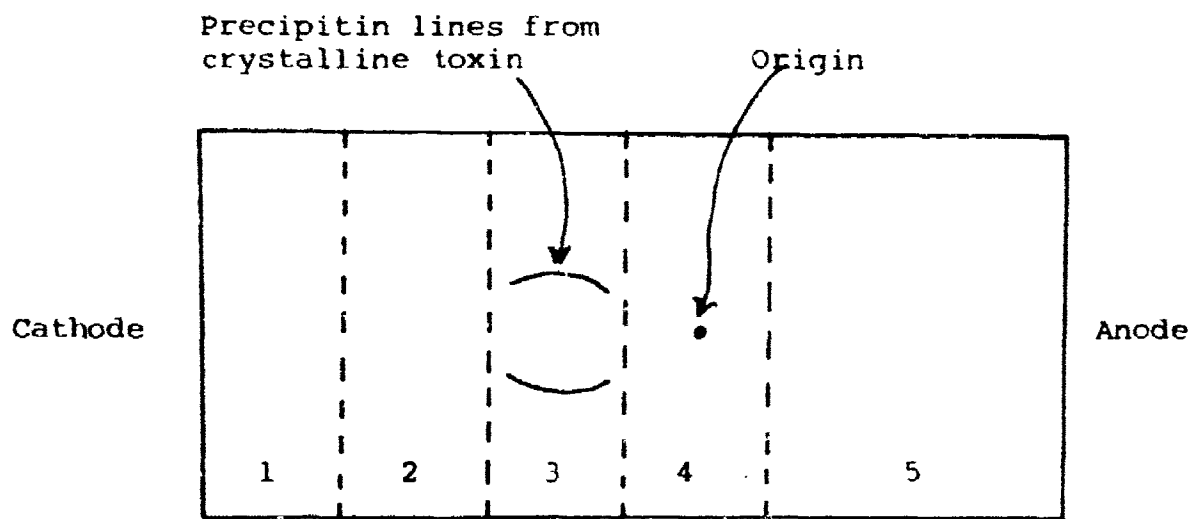
FIGURE 29

DIVISION OF AGAR SLIDE INTO SECTIONS
FOR TOXICITY DETERMINATIONS

Solutions of crystalline toxin were then fractionated by continuous-flow high-voltage electrophoresis to obtain sufficient quantities for testing. Results from a typical Elphor run of the toxin dissolved in acetate buffer, pH 3.8, are shown in figure 30. The sample at 1 mg/ml was injected into the middle of the separation chamber at 1 ml/hr and fractionated in a phosphate buffer of pH 7.4 and 0.05μ that was flowing at 100 ml/hr, with a potential of 1,800 at 180 ma. The peak fractions for toxicity and protein concentration were obtained directly opposite the input port at the middle of the chamber, which indicated that the current did not affect most of the material.

Since the fractions had been diluted by the buffer, the active fractions were pooled on the basis of precipitin and hemagglutination tests and were concentrated by dialysis against Carbowax. Figure 31 shows the schlieren patterns obtained on ultracentrifugation of the concentrate of fractions 22 through 25 and the untreated toxin at 42,000 rpm. The top pattern shows that the concentrated fractions exhibited one component that sedimented at the same rate as the single component of the untreated toxin (S_{ob} = 20.9) and another component that did not separate from the meniscus. The results of centrifugation at 60,000 rpm in synthetic boundary cells are shown in figure 32. No calculations were made for the fast component, but the S_{ob} of the slow component was 0.38.

The two components of the fraction concentrates were isolated by using the separation cells of the ultracentrifuge. The toxicities of the concentrates and the separated components are summarized in tables 13 and 14. The magnitude of toxicity of concentrates 17 through 21 and 26 through 29 was the same as the untreated toxin, but that of 22 through 25 was higher. The starting dilutions of the separated components were too high to obtain LD50 values, but the heavy component was more toxic than the light component.

Hemagglutination and precipitin tests with concentrates 22 through 25, 26 through 29, and the bottom component of 22 through 25 were positive. The same tests with concentrates 17 through 21, as well as the combined top components of 17 through 25, were negative, although these materials were neutralized by antitoxin. The toxicity of all fractions decreased on storage.

The overall results show that high-voltage electrophoresis caused dissociation of crystalline toxin into units that differ from the starting material in size, toxicity, and immunological properties.

Log dilution for 100% deaths

Absorbance at 280 mμ

Toxicity

Absorbance
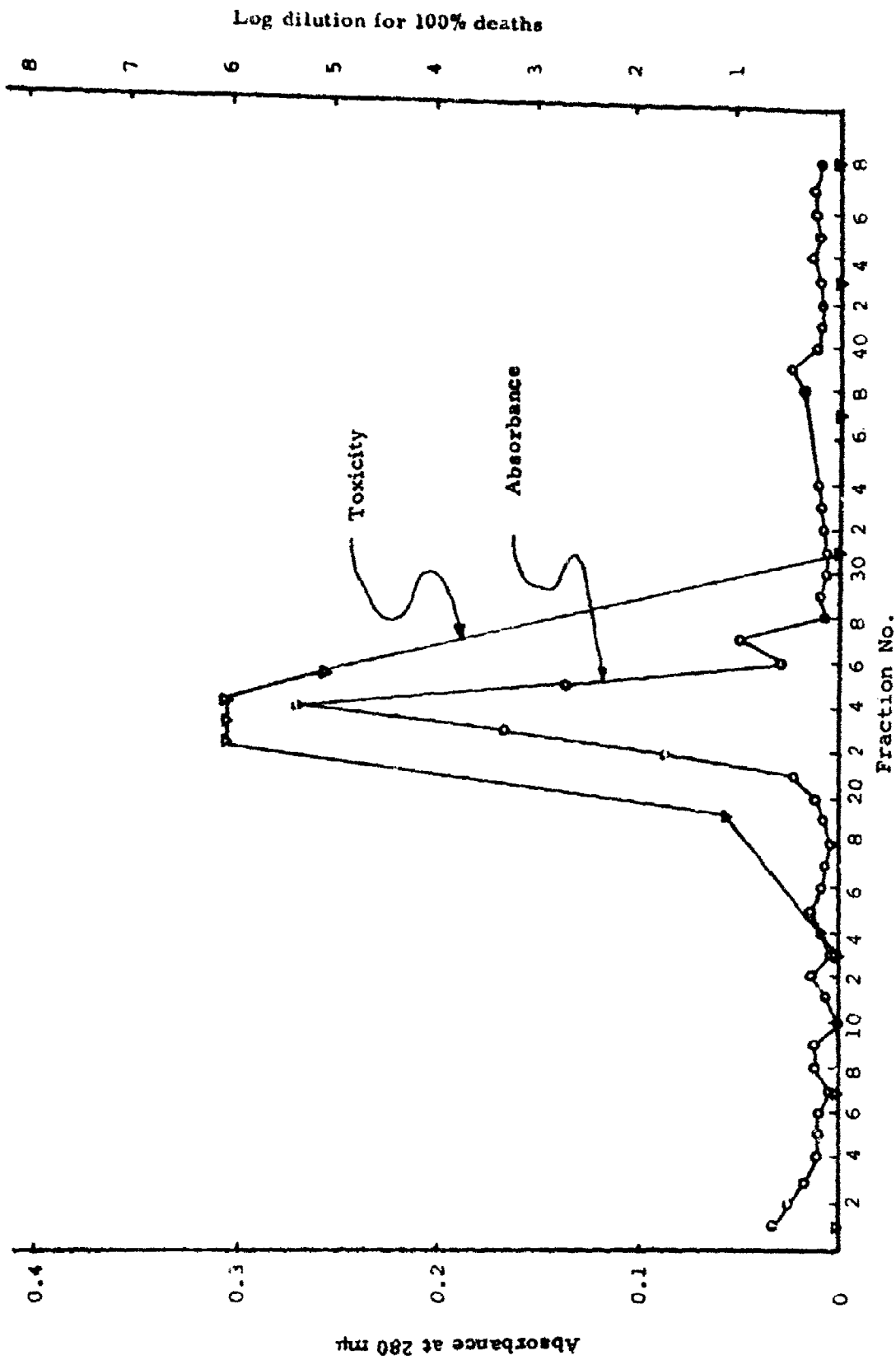
Fraction No.

FIGURE 30

TOXICITY AND ABSORBANCE OF ELECTROPHORETIC FRACTIONS

FIGURE 31

SCHLIEREN PATTERNS OF CONCENTRATED FRACTIONS 22 THROUGH 25 (TOP) AND UNTREATED TOXIN (BOTTOM) DURING ULTRACENTRIFUGATION IN ANALYTICAL CELLS

20 min
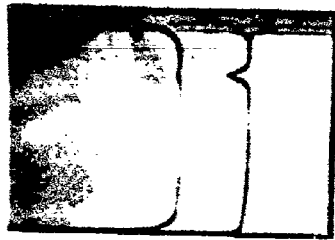
16 min
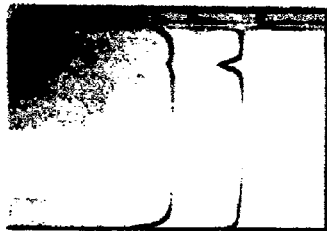
12 min

8 min

At speed: 42,040 rpm

98
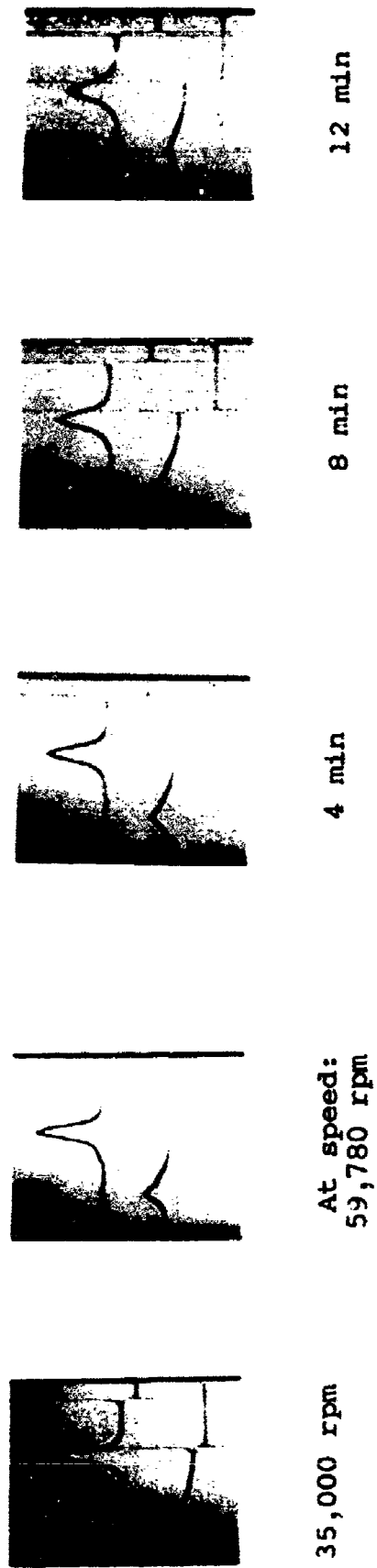
FIGURE 32

SCHLIEREN PATTERNS OF CONCENTRATED FRACTIONS 22 THROUGH 25 DURING
ULTRACENTRIFUGATION IN SYNTHETIC BOUNDARY CELLS

TABLE 13

TOXICITY OF CONCENTRATED FRACTIONS DETERMINED BY 24-HR MOUSE ASSAY

Fraction	Protein concn	Mortality fraction Dilution					
		None	1:2	1:4	1:10	1:20	1:100
	mg/ml						
Unfractionated	10.8×10^{-4}	3/4	4/4	4/4	4/4	0/4	0/4
17 - 21	2.62×10^{-4}	4/4	4/4	1/4	2/4	0/4	0/4
22 - 25	5.6×10^{-4}	4/4	4/4	4/4	4/4	4/4	2/4
26 - 29	2.60×10^{-4}	4/4	4/4	3/4	4/4	0/4	0/4

TABLE 14

TOXICITY OF SEPARATED COMPONENTS FROM CONCENTRATED FRACTIONS DETERMINED BY MOUSE ASSAY

Fraction	Component	Protein concn	Time after injection	Mortality fraction Dilution				
				$1:10^6$	$1:10^7$	$1:10^8$	$1:10^9$	$1:10^{10}$
		mg/ml	hr					
17 - 25	Top	0.47	24	7/10	0/10	0/10	0/10	0/10
			48	9/10	0/10	0/10	0/10	0/10
			72	9/10	0/10	0/10	0/10	0/10
			96	9/10	0/10	0/10	0/10	0/10
17 - 21	Bottom	0.13	24	0/10	0/10	0/10	0/10	0/10
			48	0/10	0/10	0/10	0/10	0/10
			72	0/10	0/10	0/10	0/10	0/10
			96	0/10	0/10	0/10	0/10	0/10
22 - 25	Bottom	0.47	24	10/10	10/10	1/10	0/10	0/10
			48	10/10	10/10	8/10	0/10	0/10
			72	10/10	10/10	10/10	0/10	0/10
			96	10/10	10/10	10/10	0/10	0/10

Wagman[*] obtained toxin of S_{ob} = 6.5 by dialyzing it against phosphate buffer of high ionic strength. On the assumption that pretreatment of the toxin before electrophoresis might produce a higher yield of the light component, crystalline toxin was dissolved in phosphate buffer at pH 7.5 and 0.5µ and chromatographed on Sephadex G-50 columns in a cold room. The same buffer was used for elution. The UV-absorbing fractions appeared in the eluate immediately after the void volume, indicating that the material in the fractions had a molecular weight larger than 10,000, which is the exclusion limit of Sephadex G-50. The fractions were pooled and lyophilized, and the aqueous solutions were dialyzed against Carbowax. A total of 3.3×10^{-7} mg produced death in mice in a 24-hr assay in comparison with 1.0×10^{-7} mg for the starting material.

The gel-filtered toxin and the untreated toxin were examined in synthetic boundary cells at 60,000 rpm (figure 33). S_{ob} = 0.71 for the fractionated toxin and S_{ob} = 10.50 for the fast-moving component of the untreated toxin were obtained. No calculations were made for the slow-moving component of the untreated toxin, but it appeared to sediment at the same rate as the fractionated toxin. Therefore, some preliminary fragmentation occurred in the 0.5µ buffer. The gel-filtered material produced a precipitin reaction and remained stable at 4°C.

The results obtained by the two different methods of fragmentation and other results from characterization studies are summarized in table 15. The toxicity values listed are not absolute in terms of LD50, but refer to the lowest toxin concentration that killed all the mice that were injected. The values of S_{ob} were not corrected for the viscosity of the solvent.

Preparation A (table 15) is the crystalline toxin that has a reported molecular weight of 900,000. After continuous-flow electrophoresis, preparation B was obtained, and it had a toxicity very similar to that of the original material, but it was unstable with time. The toxicity dropped to zero after storage in the refrigerator for 4 or 5 days. Two components were present in this preparation, and, after separation in the ultracentrifuge, the light component, C, was found to be about one-tenth as toxic as the heavy component, D.

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[*] Wagman, J. Arch. Biochem. Biophys. 50, 104 (1954).
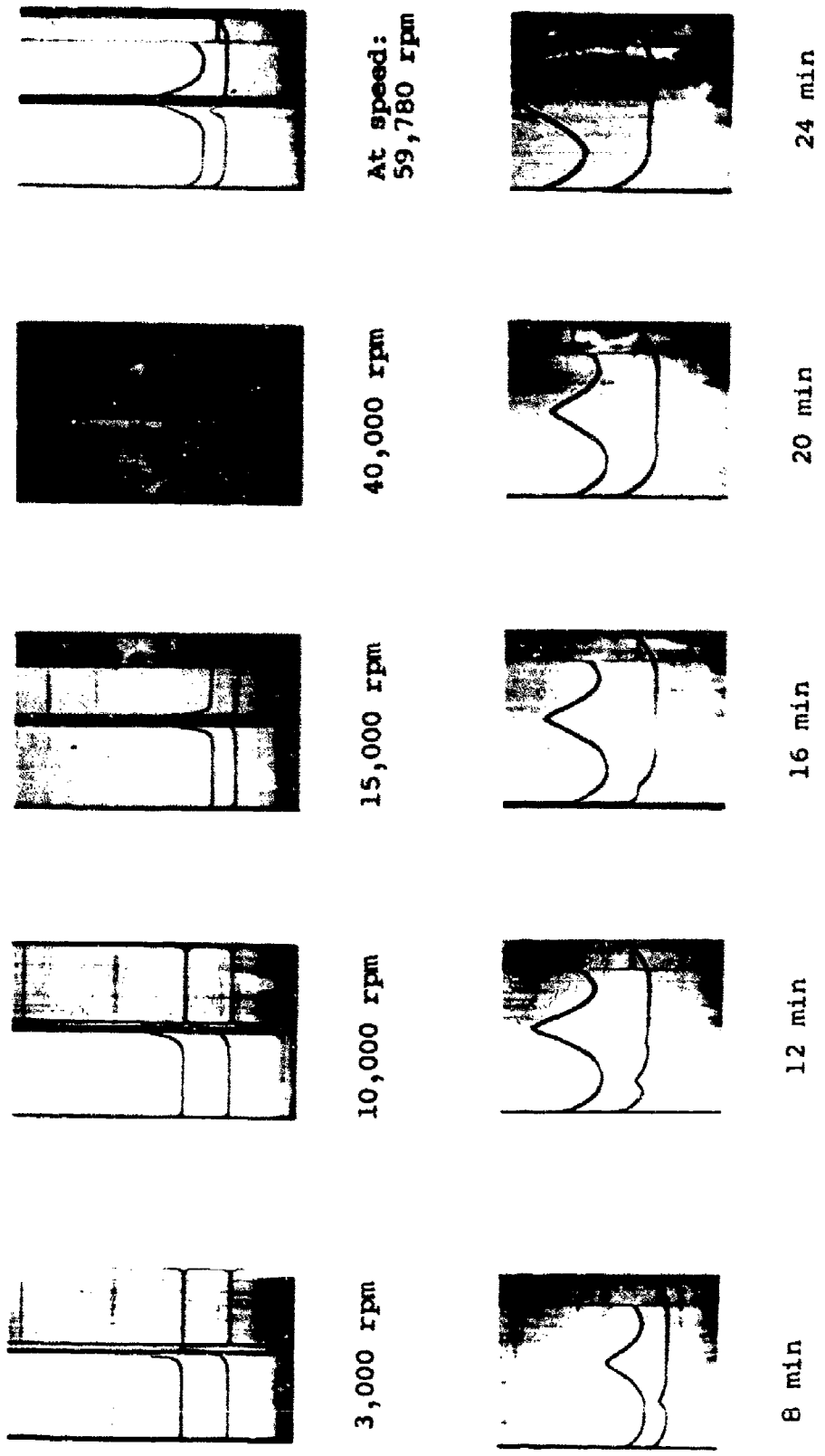
FIGURE 33

SCHLIEREN PATTERNS OF GEL-FILTERED FRACTIONS (TOP) AND UNTREATED TOXIN (BOTTOM) DURING ULTRACENTRIFUGATION IN SYNTHETIC BOUNDARY CELLS

TABLE 15

SUMMARY OF RESULTS

Toxin preparation	Toxicity* mg N	S_{ob} Heavy	S_{ob} Light	Estimated molecular weight Heavy	Estimated molecular weight Light
A (crystalline, in 0.05μ acetate)	9.5×10^{-9}	20.9	None	900,000	—
B (electrophoretic)	6.7×10^{-9}	Not measd	0.38	Not estd	<5,000
C (light component of B)	1.6×10^{-8}	None	0.38	—	<5,000
D (heavy component of B)	1.6×10^{-9}	Not measd	None	Not estd	—
E (crystalline, in 0.5μ phosphate)	2.4×10^{-9}	10.5	Not measd	200,000	Not estd
F (gel-filtered)	3.0×10^{-10}	15.2	None	700,000	—
G (Carbowax-treated F)	4.9×10^{-8}	None	0.71	—	<10,000
H (lyophilized F)	7.0×10^{-9}	None	0.65	—	<10,000
I (dialyzed H)	2.5×10^{-8}	Not measd	1.80	Not estd	>10,000
J (light component of I)	2.5×10^{-5}	None	1.84	—	<10,000
K (heavy component of I)	1.2×10^{-6}	Not measd	None	Not estd	—
L (50-L-25: gel-filtered, lyophilized, and refiltered)	1.7×10^{-9}	None	0.53	—	<10,000

* Highest dilution at which 100% death occurred.

When the crystalline to ı was dissolved in 0.5μ phosphate (forming preparation E), the toxicity changed slightly, but a two-component mixture was obtained. Some fragmentation occurred, since the S_{ob} of the largest component was 10.5.

On gel filtration of preparation E through Sephadex G-50, preparation F, which apparently was more toxic and had a larger S_{ob}, was obtained. After Carbowax treatment (preparation G) or lyophilization (preparation H), however, only one component was present in the ultracentrifuge, and the S_{ob} was quite small.

All of the gel-filtered preparations contained large quantities of salt, and when they were dialyzed against distilled water to remove the salt, other changes occurred. Preparation H, which originally contained one component, produced a mixture of two components that was less toxic than the starting material. One component exhibited a higher S_{ob}. When these components were separated in the ultracentrifuge, a further decrease in toxicity occurred. When starting with a material with a relatively high salt content that exhibits only one component, a decrease in toxicity as well as an increase in size of the molecule occurs after dialysis.

Preparation L resulted from unsuccessful attempts at salt removal by gel filtration of preparation H through Sephadex G-25 and elution with water. There was little change in toxicity, and the S_{ob} was still small; however, the preparation still contained about 80% salt.

From the results of this work, it is concluded that relatively small toxin molecules were produced by two different methods. In electrophoretic separation, the toxicity of the product is not stable, and the product differs in immunological properties from the original toxin. In the other method, some fragmentation occurs when crystalline toxin is dissolved in high-ionic-strength phosphate buffer. After Sephadex treatment and lyophilization, however, the stable, toxic, small-molecular-weight subunits that were obtained had immunological properties similar to those of the original toxin.

It is interesting to compare these results with those of other workers. Wagman* reported an irreversible dissociation of Type A toxin at pH 9.2 and 0.2μ to give components with S_{ob} values of 7.3 and 13.7.

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* Wagman, J. Arch. Biochem. Biophys. 100, 414 (1963).

The slower component was further degraded to $S_{cb} = 3.0$ to 3.6 by the action of pepsin in acetate, pH 3.8 and M = 0.05. In one experiment, dialysis of the peptic digest against five volumes of acetate produced a diffusible toxin unit. Wagman attributes the erratic results to possible contamination or to faulty membranes that might have been used.

In our experiments, dialysis was against 100 volumes of distilled water, and diffusible toxin was never found in the outer solution, although the protein content of the material always decreased in the dialysis sac. In addition, the original material, with a very high salt content, contained only one component before dialysis but two components after dialysis. Neither of the two components was as toxic as the original material, indicating that the salt content was important for both size and toxic stability.

More recently, Gerwing and coworkers* obtained a toxic unit of Type A toxin by ammonium sulfate fractionation of the toxin filtrate and subsequent elution from diethylaminoethylcellulose (DEAE-cellulose) with phosphate-citrate buffer (pH 5.6, M = 0.067). Ultracentrifugation after concentration against propylene glycol resulted in a component with an $S_{ob} = 0.93$ and a molecular weight of 12,200.

We believe that the results obtained from both methods are compatible; in fact, the two products probably are the same. If this is true, the dissociation of the large molecular into small lethal units and the isolation of a similar small unit from the cultural filtrate increase the possibility that the molecule with a molecular weight of 1,000,000 is an artifact of the preparation by aggregation of smaller units.

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* Gerwing, J., Dolman, C., and Bains, H. J. Bacteriol. 89, 1383 (1963).

DISCUSSION

Question: What is the molecular weight of your toxin?

Dr. King: We made an approach-to-equilibrium study of the material and obtained a value of 9,000 for the molecular weight. I am sure that our value of 9,000 is not as accurate as their value of 12,000, but, optimally, the subunit falls within the range of 5,000 to 10,000.

Question: Did the Sephadex change the toxin?

Dr. King: No, I do not think it changed the material; it merely aided in breaking up this artifact, if we can term it as such.

Dr. Zacks (The Pennsylvania Hospital): I noted from Dr. Riesen's paper that in recovery from a Sephadex column, he also gets about 70% of the starting material. In using labeled toxin, we also get recovery of this order, although if you use other proteins (we use albumin as a standard), you can get recovery on the order of 90%. I wonder whether this missing 20% might indicate an even smaller subunit of the toxin, and it struck me in listening to your paper and to Dr. Reisen's that it might be worthwhile for both of us to take our Sephadex columns and homogenize and dialyze them to see if we can get an even smaller component to come out of them.

Dr. King: There is something left on the Sephadex column. The material we get from the G-50 column comes off right after the void volume, and, therefore, you would expect that it would have a molecular weight larger than 10,000. The discrepancy is probably due to the fact there is so much salt associated with the molecule that the effective molecular size is larger. After running large volumes of eluate through the column, we do not obtain UV-absorbing material in the collection tubes. If the column is broken down and the Sephadex is washed with distilled water, however, a general UV absorption is obtained, not the characteristic 280-mμ maximum that is normally obtained for proteins. We have not attempted to characterize the residual material.

Dr. Riesen (IITRI): We obtained some, ranging from 10% to 60% or 70%, depending on the ionic conditions within the column. I think that repeated elution with the same ionic medium would not release the material that is on the column. It might be difficult to get this off because of its surface activity.

SESSION II

28 June 1965

Chairman: Dr. J. H. Wills
Chief, Physiology Division, CRDL

AVAILABLE FORMS OF BOTULINUM TOXIN

Dr. E. J. Schantz
Chief, Chemistry Branch
USA Biological Laboratories
Fort Detrick

Many of the contractors have requested botulinum toxin for their work. I usually prepare it for the work we do at Fort Detrick in two forms. One is a solution of the crystalline toxin in 0.05 M acetate buffer at pH 4.2 that is stable for perhaps a year with no appreciable loss in toxicity. The concentration usually runs from 3 to 5 mg/ml. To dissolve the toxin in this buffer (it is crystallized at about pH 6.8), it is necessary to go through its isoelectric point of pH 5.6. If it is then put back into a physiological buffer, it goes through the isoelectric point again. This will denature the toxin to some extent, unless it is taken through the isoelectric point very quickly. The other form in which we store the toxin is as crystals suspended in ammonium sulfate. There is some advantage in storing the toxin in the crystalline form. The crystals can be stored for more than a year with no loss in activity. The concentration of the crystals is usually 20 mg/ml. It is best to centrifuge the crystals out of this solution. They can then be dissolved in any buffer for a particular study.

If you store the toxin in an acetate solution and freeze it, it loses all its toxicity. In phosphate buffer or succinate buffer, freezing does not destroy the toxicity. Both solutions and crystals can be sent through the mail with no special precautions regarding the temperature. They are sent in a sealed glass tube contained in a sealed metal tube filled with soda lime. These tubes are placed in a cardboard mailing tube. If breakage occurs, the soda lime will destroy the toxin and render it harmless. When you receive the toxin, it should be refrigerated as soon as possible; do not freeze it, but keep it at about 2° to 4°C. The concentration of the toxin is determined by measuring the absorbance of the dissolved toxin at 278 mμ in a 1-cm cell and dividing this value by 1.67 to get the milligrams of toxin per milliliter.

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DISCUSSION

Mr. Feinsilver (CRDL): I would like to know about your experience with the 10% pure material. We have found that by freezing it at 0°F, it is stable for many weeks.

Dr. Schantz: You are talking about what we call mud?

Mr. Feinsilver: Yes.

Dr. Schantz: You can freeze it and it will probably keep for many years. We have frozen mud and kept it for a long time. Frozen in a phosphate buffer at pH 6.8, it will keep for a long time.

Mr. Feinsilver: I am sorry, I did not mean to concentrate on mud. What about the solutions of about 50,000 MU/cc?

Dr. Schantz: In phosphate buffer they will keep fine, but frozen in acetate buffer, you will have no toxicity whatever. Well, perhaps a trace, but practically none. The shaking, as it goes through the mail, will cause some slight denaturation and you will see threads in the solution. It has been our experience at Detrick that these threads cause no harm. I centrifuge them out. If the material looks milky, it was probably heated somewhere along the way and probably shaken severely. If it looks milky, do not use it. We will furnish another sample. Generally, it is pretty stable. Have you a comment, Dr. Spero?

Dr. Spero (Fort Detrick): If you work on the acid side of the isoelectric point and have to freeze the toxin, replace the acetate buffer with oxalate or succinate buffer, and the toxin will be perfectly safe.

Dr. Schantz: We have frozen it successfully in succinate so that, if you have to work on the acid side, this buffer is a possibility.

Question: Does the toxicity of the batches vary? Do you measure it for each batch?

Question: Do you measure the sedimentation rate on each batch of toxin?

Dr. Schantz: We always obtain an S value of about 17, and, by our method of preparation, it is monodisperse, with a sharp peak. Although we do not know that the crystalline product is all toxin, it seems to have a constant composition at all times. The absorbance of the toxin is 16.7 for a 1% solution measured in a 1-cm cell at 278 mμ. If the ratio of the absorbance at 260 mμ to that at 278 mμ is less than 0.55, the toxin can be considered quite pure. Even at a ratio of 0.6, the impurity (mostly nucleic acid material) would be less than 1%.

IMPORTANT QUESTIONS IN BOTULINUM RESEARCH

Dr. J. H. Wills
Physiology Division
CRDL

There are 10 questions that are particularly important in botulinum research. One is how can the intoxication be detected soon enough so that an intoxicated person can be treated effectively by the available means? The general purpose of our contract program is to find out how to treat the poisoning, not just with antitoxin, but with something that would be more widely effective than the antitoxin over a longer period of time. We have to find out a number of things about what the material does and how it behaves in the body and other things that relate to the changes it induces.

Other questions are through what part of the gastrointestinal (GI) tract is the toxin absorbed, and how is the toxin conveyed across the GI epithelium after it is ingested. We must discover not only the mechanism of transport, but the form of the material in transport. Is it transported as a depolymerized unit, or is it transported as a large polymer? How is the absorbed toxin disseminated throughout the body? It is transported by the blood, but we still need to know in what form it exists in the blood and whether it is carried as some kind of a complex, perhaps as a glycoprotein.

Another puzzle is why are early signs of poisoning confined to the head. The answer may, perhaps, be related to the similar question relating to myasthenia gravis.

A further important point is the significance of the lag period. Is this simply a period during which depolymerization takes place, or is it a period during which the material has to penetrate various barriers to its site of action? How does the toxin interfere with the function at neuromuscular or other neuroreceptor junctions? We say that it interferes by decreasing the release of ACh, but, how does the toxin interfere with the release of ACh?

Next, what is the effect on nerve endings that slows regrowth? Josephson and Thesleff recently found that poisoning with botulinum toxin had exactly the same effect on the properties of skeletal muscle as denervation. We have heard from Dr. Brooks that muscle paralyzed with botulinum toxin is still reactive to ACh. This is true, but its reactivity is different from that of normal muscle. It differs in this way: The whole surface of the muscle fiber becomes reactive to ACh; the reactivity to ACh is no longer restricted to the region around the end plate, as it is in normal fibers. Josephson and

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Thesleff also found that when they poisoned a nerve ending with botulinum toxin, it took this nerve junction a long time to become active again. If, however, they brought a new nerve fiber into the muscle, it could form an end plate on the muscle very quickly. The muscle could become innervated by the new nerve fiber while the old fiber was still ineffective in transmitting nerve excitation. These facets will be discussed by Dr. Drachmann of Tufts Medical School.

The ninth question relates to some work by a Russian author named Matveyev, which appeared in 1959. Matveyev found that if he poisoned a variety of animal species (rabbits, guinea pigs, and rats) by giving them daily doses of one-hundredth of the acute lethal dose of botulinum toxin, the animals died after 2 to 11 doses. What do repeated administrations of these small doses of botulinum toxin accomplish that makes the toxin, given in this manner, 10 to 50 times as lethal as a single dose?

The final question, the one in which our real interest lies, concerns the means by which the lethal actions of botulinum toxin can be antagonized. Here, we refer to means other than the immunological approach—to chemicals that can be made easily and readily. There is little available information on this question. In 1948, Lewis and Page reported that desoxycorticosterone acetate (DOCA) was effective in protecting adrenalectomized rats from lethal doses of the toxin. In the same year, Miller and Nelson reported that general anesthetics and two amino derivatives delayed the lethal effects of the toxin. Gossweiler, in two papers published in 1957, reported that certain dinitriles (particularly succinodinitrile) and 1,2-bis(ethylamino)propionitrile have some ability to alter mortality from the toxin in goldfish and guinea pigs. We hope, in time, to possess more effective chemicals than these for the treatment of botulinum toxin poisoning.

POSSIBLE SITES OF ABSORPTION AND DISTRIBUTION
OF BOTULINUM TOXIN

Dr. D. M. Serrone
Albany Medical College

These studies represent a number of experiments performed at the Institute of Experimental Pathology and Toxicology, Albany Medical College.

Table 16 represents the LD50 values obtained with botulinum toxin during the past year. The LD50 values were calculated by the method of Miller and Tainter, and 72-hr LD50 values are given. Female, albino, Swiss-Webster mice (18 to 22 gm) were used. Two grams of crude toxin, brought up to 100 ml with phosphate buffer, were used. We experienced some decay in the potency of the toxin over the 2-yr period. No difference was observed in the toxicity between male and female mice when the toxin was given orally. Oral administrations of the toxin proved to be 10,000 times less toxic than ip administrations.

The LD50 in male rats was expressed in terms of mouse LD50 (MLD50) units. A single ip injection resulted in 7.6 ±1.8 MLD50 units/100 gm of body weight, whereas three consecutive ip injections resulted in 6.1 ±2.3 MLD50 units/100 gm of body weight. Three consecutive daily administrations of the toxin did not appear to be more toxic than a single administration.

The second sample of the crude toxin was obtained and had the same range of toxicity as the first sample. Assay of the crystalline toxin was performed in mice, and the results were expressed in terms of the original solution of toxin.

The various orally administered agents used in an attempt to block the absorption and, possibly, the lethal action of the toxin are listed below.

Colchicine salicylate	1.5 mg/kg
Okra mucilage, 2%	0.2 ml/mouse
Parathion	4.0 mg/kg
Carbaryl	300 mg/kg
Iodoacetate	40 µg/kg
Corn oil	0.2 ml/mouse
Fasting	24 hr
Methionine	200 mg/kg
Ethionine	200 mg/kg
Ethanol, 20%	0.2 ml/mouse

TABLE 16

LD50 VALUES IN MICE CALCULATED 72 HR
AFTER ADMINISTRATION OF CRUDE OR
CRYSTALLINE BOTULINUM TOXIN

Date/64	Route	Sex	LD50 values
			$\times 10^{-5}$ µg/mouse

A. Crude Toxin (First Sample)

Date/64	Route	Sex	LD50 values
5/7	Ip	F	4.4 ±0.63
5/13	Ip	F	8.2 ±0.65
5/18	Ip	F	6.2 ±0.40
6/3	Ip	M	24 ±2.2
6/10	Ip	F	26 ±3.4
6/24	Ip	M	40 ±6.1
7/22	Ip	M	40 ±3.9
7/15	Po	F	485,000
10/6	Ip	F	15.5 ±3.4
10/13	Ip	F	48.5 ±9.6
11/3	Ip	F	5.8 ±1.1
11/24	Ip	F	47.0 ±4.4
12/12	Ip	F	1.6 ±0.37

B. Crude Toxin (Second Sample)

Date/64	Route	Sex	LD50 values
2/9	Ip	F	< 1
2/16	Ip	F	1.06 ±0.26
2/23	Ip	F	Ca. 10

C. Crystalline Toxin

Date/64	Route	Sex	LD50 values
11/3	Ip	F	16*
11/24	Ip	F	4.6*

* Values are $\times 10^{-10}$ ml, expressed in terms of
original solution of toxin (Type A) in 0.9 M
ammonium sulfate, obtained from CRDL.

Colchicine � ʟicylate was given in two treatments, 4 and 24 hr,
before the administra᷄ on of the toxin. Colchicine salicylate had been reported
to inhibit enzymes of ᴜᴇ GI mucosa (as measured by histochemical tech-
niques). This treatment might either enhance or block intestinal absorption
of the toxin.

A mucilage of 2% okra, which would coat the GI tract, might
thereby prevent absorption of toxin. Parathion and carbaryl, known cholin-
esterase inhibitors, might antagonize the action of the botulinum toxin.
Iodoacetate, which has also been reported to inhibit intestinal enzymes, might
either facilitate or prevent absorption. Corn oil provides an ᴏil base in the
intestinal tract that could either prevent or aid absorption of the toxin.

A 24-hr fast was imposed to provide a relatively clean intestinal
tract for administration of the toxin. Methionine and ethionine, two amino
acids, were used as possible antagonists to the absorption of the toxin. A
20% ethanol solution was used to test the theory that alcohol might prevent
absorption of toxin.

The results indicated that the corn oil and fasting appeared to
increase mortality, whereas ethanol pretreatment appeared to decrease
mortality. Pretreatment with the other agents did not alter mortality.

Another experiment was designed to study absorption of the toxin
and its movement through the intestine. Belco parabiotic tissue-culture
tubes were prepared so that a segment of intestinal tract could be inserted
between the halves. Toxin (10,000 MU) was added to one side of the tissue.
The tubes on both sides were filled with Kreb's Ringer solution (14 ml), and
the preparation was incubated for 1 hr at 25°C. After incubation, the fluid
was removed and assayed by ip injection into mice.

These experiments indicated that the toxin can pass through the
stomach in either direction—mucosal to serosal or serosal to mucosal
(table 17). There was little or no transport from the mucosal to the serosal
side of the duodenum, jejunum, or ileum. There was some transport from
the mucosal to the serosal side of the colon.

Effect of Toxin on Enzyme Systems.

A series of experiments was conducted in albino rats to ascertain
the effect of the toxin on hepatic demethylase activity and on brain and plasma
cholinesterase levels. Rats were injected ip with 2 or 4 MU of toxin/100 gm of
body weight. They were sacrificed for study 24, 48, and 72 hr after adminis-
tration of the toxin.

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TABLE 17

TRANSPORT OF TOXIN THROUGH GI TRACT IN VITRO

Organ	Mucosal/serosal	Serosal/mucosal
Stomach	+	+
Duodenum	-	—
Jejunum	-	—
Ileum	-	—
Colon	±	—

As shown in table 18, demethylase activity was measured in a liver fraction containing microsomes and supernatant (9,000 g's); propoxy-phenecarbinol was the substrate, and the formaldehyde produced was trapped as the semicarbazone and assayed using Nash reagent.

TABLE 18

EFFECT OF BOTULINUM TOXIN ON RAT-LIVER DEMETHYLASE ACTIVITY

Time after admin	No. of rats	Ratio of HCHO:liver
hr		μmoles:gm
A. 2.0 MLD50 Units*		
24	4	4.37
48	4	4.12
72	4	4.77
Control	6	4.70
B. 4.0 MLD50 Units*		
24	4	3.77
48	4	3.46
72	4	5.21
Control	6	5.21

* Per 100 gm of body weight, ip.

Demethylase activity did not change following the administration of 2 MU/100 gm of body weight. Following the administration of 4 MU, however, demethylase activity was depressed at 24 and 48 hr but appeared to recover by 72 hr. Rats receiving 4 units demonstrated marked symptoms at 24 and 48 hr (labored breathing, etc.), but were recovering by 72 hr.

Cholinesterase activity was measured with the Warburg apparatus using ACh bromide as the substrate. Neither brain nor plasma cholinesterase activity was changed following ip administration of 4 MU/100 gm over a 72-hr period (table 19).

TABLE 19

EFFECT OF BOTULINUM TOXIN ON RAT-BRAIN
AND PLASMA CHOLINESTERASE ACTIVITY

Time after admin	Group	Cholinesterase activity	
		Plasma	Brain
hr		μl of CO_2/20 min	
24	Control	47.7	172.3
	Treated	49.3	199.8
48	Control	45.9	154.3
	Treated	42.4	179.0
72	Control	46.8	196.3
	Treated	42.4	166.6

Negative-Phase Studies.

These studies were undertaken to elucidate the fate of the toxin between the time of injection and the appearance of toxic symptoms. If, in these hours (sometimes days), there was accumulation in an organ, studies in depth could be concentrated on this target organ. The principle was the same as that used by Coulston and his colleagues in studies of the distribution and localization of malarial parasites.

Large doses of toxin (10, 000 or 50, 000 MU) were injected iv via a tail vein into adult, Sprague-Dawley rats. Rats were sacrificed at varying times, and blood and tissue samples were taken. Homogenates of various organs were prepared in a phosphate buffer and assayed by ip injection into mice (0.25 ml).

Rats were given 10,000 units iv and killed 1/2 hr later. This dose would ordinarily kill the rats in about 4 hr. The mortality data in table 20 are for the mouse assay. Neither of the mice injected with serum was dead 24 hr following injection, but both had died by 72 hr. One mouse injected with kidney homogenate died in 48 hr. These were the only deaths.

TABLE 20

DISTRIBUTION OF BOTULINUM
TOXIN IN RAT (10,000 UNITS IV)
SACRIFICED AT 1/2 HR

Sample (rat)	Mortality fraction (mice)		
	24 Hr	48 Hr	72 Hr
Serum	0/2	0/2	2/2
Brain	0/2	0/2	0/2
Lung	0/2	0/2	0/2
Liver	0/2	0/2	0/2
Kidney	0/2	1/2	1/2

We became interested in the blood distribution of the toxin, and 50,000 MU were injected iv into a rat. Samples were taken at 1/2, 1, 2, and 4 hr. The blood was collected in heparinized tubes and centrifuged to separate erythrocytes from plasma. Serial dilutions in saline were made with plasma (1:10, 1:100, and 1:1,000). The red blood cells (RBC) were washed three times with saline and resuspended in saline. A portion of them was hemolyzed in distilled water. The + signs in table 21 indicate that symptoms of toxicity were observed in the mice used for bioassay at this time interval. The mortality is given for the 72-hr deaths in mice given the assay material ip.

Undiluted plasma was lethal to all mice at all time intervals. The 1:10 plasma dilution was lethal to all mice except at 4 hr, when it killed 2/4. The 1:100 plasma dilution did not kill any mice, but symptoms were observed at all time intervals except the 4-hr period. No deaths or symptoms were found with the 1:1,000 dilution.

TABLE 21

MORTALITY OF MICE INJECTED WITH MATERIAL FROM RATS POISONED WITH BOTULINUM TOXIN

Sample (rat)	Mortality fraction (mice)			
	1/2 Hr*	1 Hr*	2 Hr*	4 Hr*
Plasma	4/4	4/4	4/4	4/4
1:10	4/4	4/4	4/4	2/4
1:100	0/4+	0/4+	0/4+	0/4
1:1,000	0/4	0/4	0/4	0/4
Washed RBC	0/4	0/4	0/4	0/4
1st wash	2/4	4/4	4/4	4/4
2nd wash	0/4+	0/4	4/4	1/4
3rd wash	0/4	0/4+	0/4+	0/4+
Hemolyzed RBC	0/4	0/4	0/4	0/4

* Plus signs indicate symptoms of toxicity.

Injections of washed RBC produced no symptoms at any time, and hemolysis of the washed cells did not alter the result. The first wash was lethal at all intervals, but only to the extent of 2/4 at 1/2 hr; it was 100% lethal thereafter. The second wash resulted in symptoms at 1/2 and 1 hr, but no deaths. At 2 hr 4/4 died, and at 4 hr 1/4 died. The third wash produced symptoms at 1, 2, and 4 hr.

The results of these experiments indicated that the toxin remains in the fluid portion of the blood. Some toxin, however, may be adsorbed on the surface of the RBC, but can be removed by washing with saline. There is no apparent penetration of the toxin into RBC up to 4 hr. From additional experiments, we know that clotting of the blood does not remove the toxin from the fluid portion, as it is still present in serum.

The experiments suggest that there is a decrease in the blood level at 4 hr. At this time there was 50% mortality with the 1:10 dilution of plasma, whereas this dilution was 100% lethal at previous intervals. There was also an absence of symptoms produced by the 1:100 dilution at 4 hr.

When the results produced by injection of the washings of the RBC were analyzed, it appeared that peak absorption of the toxin on the cells occurred at 2 hr. At this interval, the second washing was 100% lethal, and it was still 25% lethal at 4 hr. Apparently, there was accumulation of the toxin on the surface of the RBC during the first 2 hr; then, with a decrease in blood level, this accumulation was reduced.

In another experiment, the results of which are given in table 22, rats were given 50,000 MU iv and sacrificed at 2 and 4 hr. Mice were injected with the rat tissue, and their mortality was recorded. There was a decrease in concentration of toxin in the plasma at 4 hr, as seen in the 1:10 plasma dilution. This also occurred in the previous experiment.

TABLE 22

MORTALITY OF MICE INJECTED WITH MATERIAL
FROM RATS POISONED WITH BOTULINUM TOXIN

Sample (rat)	Mortality fraction (mice)		
	24 Hr	48 Hr	72 Hr
A. Rats Sacrificed at 2 Hr			
Plasma	4/4	4/4	4/4
Plasma (1:10 dilution)	4/4	4/4	4/4
Liver	1/4	4/4	4/4
Kidney	3/4	4/4	4/4
B. Rats Sacrificed at 4 Hr			
Plasma	4/4	4/4	4/4
Plasma (1:10 dilution)	1/4	2/4	2/4
Liver	0/4	3/4	3/4
Kidney	3/4	4/4	4/4

The kidney seemed to have a greater concentration of toxin than the liver at both 2 and 4 hr. The concentration of toxin in the liver appeared to be less at 4 hr than it was at 2 hr.

Additional attempts to extrapolate the time curve by using the oral route of administration are in progress.

DISCUSSION

Dr. Stemmer (University of Cincinnati): Did you isolate any of the blood content of the liver and kidney with regard to toxicity?

Dr. Serrone: We attempted to do all we could to rid the organs of blood. We flushed them with saline to eliminate as much residual blood as possible, so that this could not contaminate them. Certainly, there is some blood left, especially in the liver. We would expect to find a great deal more in the liver than in the kidney. This is encouraging because you will find more of the toxin contaminating the liver than the kidney.

LOCALIZATION OF BOTULINUM TOXIN, WITH SOME REMARKS ON THE BINDING OF LEAD AT MOTOR END PLATES

Dr. M. Sheff
The Pennsylvania Hospital

A simple account of the methods used to produce a fluorescein-labeled botulinum toxin will be presented.

The crude material was examined by disk electrophoresis, and, as previously noted in one of our reports, many components were found. Furthermore, the toxicity extended all the way down the gel; it was not limited to the stainable protein components. It was decided, therefore, to first attempt to label whole crystalline botulinum toxin using a standard system. The labeling system has been described by Rinderknecht.* Essentially, it consists of dissolving the protein in a buffer at pH 8 and at fairly high ionic strength, adding fluorescein or rhodamine on Celite to this, letting the mixture react for 3 min at room temperature, spinning off the Celite, and separating the free dye in the supernatant from the labeled protein on a Sephadex G-25 column. We were familiar with Wagman's studies, and, according to our interpretation of his 1961 paper, dialysis for 14 hr seemed to be required to split the crystalline toxin into smaller components. Splitting the toxin was thought to be unnecessary under the alkaline conditions and high ionic strength that occur during the 3-min period of labeling before the toxin is put on the Sephadex column for separation from the free dye, when the pH is reduced. As the protein and the free dye are eluted from the column in pH 6.8, 0.02 M phosphate buffer at 4°C, the fractions were expected to be relatively stable. The results of this, however, were rather discouraging. Several peaks were obtained. A large amount of material came down in the void volume of the column and started to precipitate while being collected. The other fractions that came off were not fully separable. These results indicated that the toxin had undergone considerable splitting during its preparation.

It was decided, therefore, to make use of the dissociation of the toxin by buffers at relatively high pH to see if a characterizable set of products to use in the labeling experiment could be produced. The crystalline material was incubated at pH 8.3 in 0.5 M carbonate-bicarbonate buffer for varying periods. There was very little difference if incubation was for 3 hr or overnight. The product was a turbid solution that gave small amounts of precipitate, and this left a toxic solution that could be labeled. Using this

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* Rinderknecht, H. Nature 143, 167, 168 (1962).

material, column-chromatographic studies were run without a fluorescent label on the toxin, and three fractions were obtained from the column. The first of these came out shortly after the void volume and contained, presumably, the largest molecule. From ancillary studies with Sephadex C-50, G-75, and G-100, the molecular weight was thought to be on the order of 70,000 to 150,000; however, unless the molecule is spherical, elution from Sephadex columns cannot be used as a measure of molecular size. The smaller component ran at the tail of the column and probably is similar to the component with a molecular weight of 5,000 to 10,000 that other workers spoke of.

When these fractions were put into the spectrophotometer, spectral curves were obtained that looked slightly different. The results are shown in figure 34. All spectra were run at pH 13. The first fraction to be eluted (A, figure 34) showed a characteristic peak at 240 mμ and also an absorption in the 280- to 290-mμ band. A component (B) was then eluted in a very small volume that showed a peak in the peptide area (215 mμ), nothing at all at 240 mμ, and hardly any absorbance at 280 mμ. Following that, another peak (C) showed peptide absorbance, a slight peak at 240 mμ, and some absorbance at 280 mμ.

We then studied the toxicities of these fractions. As expected, the toxicity extended all the way through the column. The first fraction (A, tubes 4 to 6) was trichloroacetic acid (TCA)-precipitable; the other fractions (B, tube 8, and C, tube 10) were not TCA-precipitable.

Table 23 records the relative toxicities of the fractions obtainable by chromatography on Sephadex G-25 of botulinum toxin that has been dissociated. The dissociation was performed by incubating the toxin in pH 9.2, 0.5 M bicarbonate buffer. The chromatogram was developed with 0.02 M, pH 6.8 phosphate buffer. The absorbances were measured in the spectrophotometer after suitable dilution of the fractions and the crystalline toxin with N/25 NaOH. The toxicity was measured by a standard time-to-death assay in mice using suitable dilutions of the crystalline toxin as the standard. The calculated toxicity was obtained from the simple numerical ratio of the absorbances of the fractions to the absorbances of crystalline toxin at the given wavelengths.

The remainder of the tubes from this experiment showed minimal toxicity. Aliquots of 2 ml were collected. Of a total of 20 mg of toxin as starting material, approximately 14 mg was collected in the fractions, giving a recovery of 70%. The precipitate that formed during dissociation had minimal toxic activity.

Wavelength, mμ
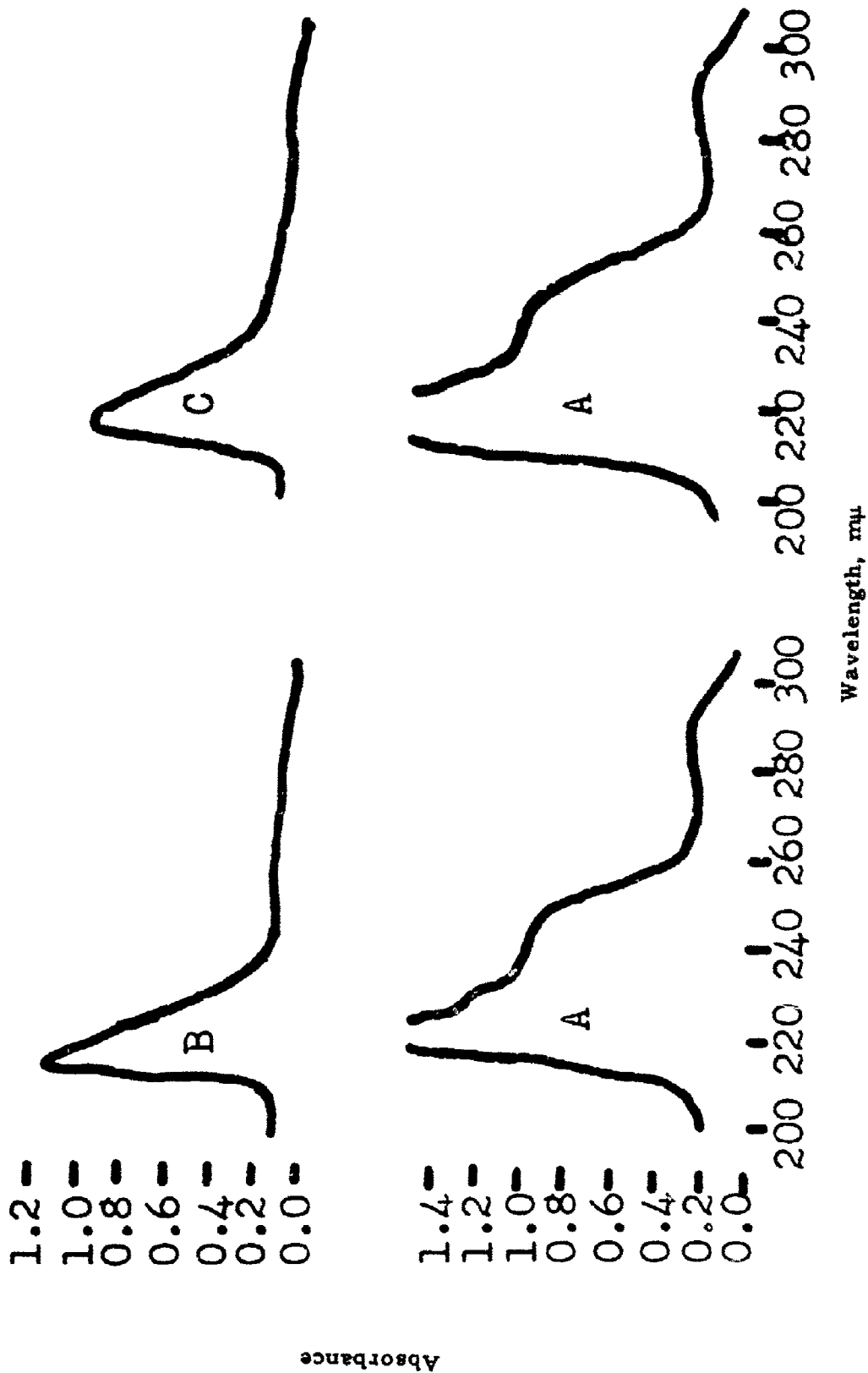
FIGURE 34

SPECTRAL CURVES OF FOUR FRACTIONS OF BOTULINUM TOXIN

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TABLE 23

TOXICITY OF FRACTIONS OBTAINED FROM CHROMATOGRAPHY OF DISSOCIATED TOXIN ON SEPHADEX G-25

Tube No.	Toxicity (a)*	Calcd toxicity*		Toxicity found	
		At 285-mμ absorbance (b)	At 215-mμ absorbance (c)	a/b	a/c
				% of calcd	
1 - 3	no toxicity	no protein			
4 ⎫ TCA-	2,000	2,400	1,860	83	107
5 ⎬ pptable	3,200	3,900	2,700	82	118
6 ⎭	1,200	800	730	150	164
7 ⎫ not	200	250	420	80	47
8 ⎬ TCA-	120	100	600	83	17
9 ⎪ pptable	80	100	370	80	22
10 ⎭	25	50	50	50	50

* Given as μg of equivalent crystalline toxin.

The nitrogen content of the crystalline material was measured and was multiplied by 6.25 to obtain the protein content. Then, parallel assay tests were performed in which groups of mice were given standardized doses of the test material and the crystalline material, thereby obtaining parallel curves of times to death. Equal times to death were an indication of equivalent amounts of crystalline toxin. Comparison of these toxicity figures to the absorbance showed that, in some respects, they were parallel to the absorbance at 215 mμ, but with one exception. This exception was the fraction (tubes 8 and 9) that did not show a peak at 240 mμ. In contrast, the absorbance at 280 to 290 mμ was more closely related to the relative toxicity. In relation to the nonspecific peptide peak, which is, under these conditions, a measure of total protein concentration, the peak of tubes 8 and 9 was less toxic than the others.

To determine the nature of the peak at 240 mμ, equal aliquots were taken; one was adjusted to pH 6 and one to pH 13, and a difference spectrum was run. Tyrosine and tryptophane both show difference spectra under these conditions, and the spectrum that was obtained is similar to that of tyrosine. It is, therefore, possible that the fractions with a peak at 240 mμ are high in tyrosine.

The toxin was labeled with rhodamine, and the amounts of label attached to the various fractions were measured. All the fractions except the free rhodamine were toxic. The absorbance at 575 mμ (the absorbance maximum for rhodamine) was compared with the absorbance at 215 and 280 mμ. The results are shown below, with the relation between the absorbances expressed as a ratio. The most marked variation is shown by fraction 8, which picked up a very large amount of rhodamine, indicating that this molecule had many free amino groups on its surface. The other fractions behaved in a manner quite similar to that of albumin. A twofold difference in the ratio is not terribly significant under the stated labeling conditions.

The relative amount of rhodamine bound to albumin is shown for comparison. The ratio is obtained by measuring the absorbance of the solution at 575 mμ and comparing it with the absorbance at 215 mμ (the generalized peptide peak) and at 280 mμ (the tyrosine peak). The higher the ratio, the greater the amount of label that is attached.

	$\dfrac{\text{Absorbance at 575 mμ}}{\text{Absorbance at 215 mμ}}$	$\dfrac{\text{Absorbance at 575 mμ}}{\text{Absorbance at 280 mμ}}$	Relative toxicity*
Albumin	0.08	0.35	—
Fractions**			
1 to 3	No toxicity or protein		
4	0.01	0.10	4+
5	0.03	0.18	4+
6	0.05	0.33	4+
7	0.06	0.51	3+
8	0.14	1.20	1+
9	0.07	0.39	3+

* Relative toxicity only was estimated on this particular run because of the small amount of material remaining after other studies had been made.

** Fractions of dissociated toxin eluted from a G-25 Sephadex column after labeling.

Fractions 4 through 7 are TCA-precipitable; the rest are not. Fraction 8 is an intermediate fraction that is apparently highly positive in nature, has a low aromatic acid content, and is relatively nontoxic. Fraction 9 is a low-molecular-weight, highly toxic fraction.

In summary, three materials were obtained from the column, two of which were highly toxic. One of these is a fairly large molecule and one is a fairly small molecule. A third material, a fairly small molecule that picked up much of the dye, apparently was different from the other members of the group and was relatively nontoxic.

Figure 35 summarizes the whole system. Dr. Zacks will show in the morphological data the specificity of binding, the amount of binding, and the relative specificity of binding of fractions A and C in tissues.

One of the questions that now arises relates to the toxicity of these materials. There were one or two curiosities. The lag period was mentioned by Dr. Wills. Although parallel curves of toxicity can be drawn for these fractions, it is noticeable that with the smaller molecules the minimum time to kill an animal is considerably reduced as compared with the minimum time for death for the larger molecules. This may have some bearing on whether the molecule has to be dissociated first or whether this is another physiological variable. This aspect of the work is a preliminary to the morphological study that Dr. Zacks will discuss.

Toxin crystals incubated in 0.5 M, pH 9.2 carbonate-bicarbonate buffer

Ppt

Precipitate removed by centrifugation at 14,000 g's

Supernatant

Supernatant retained

and

labeled by the addition of rhodamine B on Celite

PO₄

Celite removed by centrifugation, and products of labeling transferred to G-25 Sephadex column for elution with pH 6.8, 0.02 M phosphate buffer

Supernatant

Free dye moves very slowly

A ────── Nonprecipitable fraction runs just before the dye (shows a high tyrosine content, high toxicity)

B ────── This fraction appears to be low in aromatic amino acids and has a relatively low toxicity

C ────── Most of this fraction is precipitable with TAC; has relatively high tyrosine and toxicity

"A" Binds strongly to motor end plates, with less generalized tissue binding than "C"

"C" Binds strongly to motor end plates, with some generalized tissue binding

FIGURE 35

DIAGRAMMATIC SUMMARY OF DISSOCIATION AND LABELING OF BOTULINUM TOXIN

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PRELIMINARY ISOLATION OF BOTULINUM TOXIN ACTIVITY
FROM MUSCLE TISSUE

Dr. R. Sowinski
Albany Medical College

Animals given lethal doses of botulinum toxin die by asphyxiation resulting from muscular paralysis. Studies by several investigators indicate that the neuromuscular junction is the site of action. In the studies presented here, the toxin was separated, by column chromatography, from muscle tissue obtained from rats that succumbed to large iv doses of toxin.

Rats were given, by iv injection, either 10,000 LD50 MU of crude toxin or a 1:20 dilution of crystalline toxin that had 1×10^{10} LD50 MU/ml. Following these large doses, the rats died in 3 to 5 hr. After death, skeletal muscle was taken from all parts of the body and homogenized.

In addition, normal skeletal muscle was also homogenized and run through the column for determination of the normal pattern.

Preparation of Muscle Tissue for Column Chromatography.

Weighed muscle tissue was homogenized in a Waring Blendor kept in an ice bath for 1/2 hr, with rest periods to ensure that the mixture was cold. The initial volume of phosphate buffer (0.1 M = pH 7.3) that was added was approximately one-fourth to one-third the total volume. The volume at the close of the homogenization procedure was a 10% w/v mixture. Since it was still possible that the muscle cells were not broken up entirely, the homogenate was hand-milled in a Potter-Elvehjem homogenizer.

The homogenate was filtered through cheesecloth and spun at 9,000 g's in a refrigerated centrifuge. The precipitate was discarded and the supernatant transferred to other centrifuge tubes and spun at 145,000 g's for 1 hr in the cold. The supernatant, which was essentially free of particulate matter, was frozen and lyophilized. The dry powder was stored in a freezer at -20°C until ready for use.

Procedure of Column Chromatography.

Approximately 1 gm of the dry powder was dissolved in 10 ml of phosphate buffer (0.1 M = pH 7.4) and dialyzed against this buffer for 24 hr with at least two changes of dialyzing medium. The volume of the dissolved muscle-tissue powder increased to approximately 15 ml during this period. Only 10 ml

was placed on a column of DEAE-Sephadex A-50 (coarse). The eluant consisted of a three-step gradient. The top buffer solution (0.1 M buffer = pH 4.50 plus 10% NaCl) slowly dropped into the middle buffer solution (0.1 M = pH 4.50), which, in turn, dropped into the bottom buffer solution (0.01 M = pH 7.40).

Results.

A typical elution pattern is shown in figure 36. The pattern was monitored at 280 mμ. The normal tissue gave at least eight peaks. The greater portion of the second peak was found to be myoglobin. The other peaks were not identified. In order to locate the position of the botulinum toxin as it comes out of the column, purified botulinum toxin was placed on the column alone and peaked at the 350-ml volume.

Biological Assays in Mice.

The following toxin assays were made by ip injection in mice:

1.   The dry powdered muscle tissue from a rat that died following an iv injection of crystalline toxin (diluted 1:20) eluted from the column at 350 ml—2/3 mice died in 48 hr and the other within 72 hr.

2.   Botulinum toxin alone (350-ml fraction)—3/3 mice died within 18 hr.

3.   Normal muscle tissue to which botulinum toxin was added after homogenization (350-ml fraction)—3/3 mice died within 18 hr.

4.   Normal muscle tissue (entire homogenate)—0/3 mice died.

Discussion of Results.

When botulinum toxin alone was put through the column, the toxin peaked at the 350-ml volume. Recovery of the toxic fraction from skeletal muscle of a rat that had died as a result of a large iv dose of toxin was made at the same position. Mouse assay of this fraction indicated that the activity was not destroyed by the intensive homogenization required to prepare the muscle specimen.

Top: 0.1 M PO$_4$= at pH 4.50 + 10% NaCl
Middle: 0.1 M PO$_4$= at pH 4.50
Bottom: 0.01 M PO$_4$= at pH 7.40

Column: DEAE-Sephadex A-50 (coarse)

Absorbance in arbitrary units at 280 mµ

Volume of effluent, ml
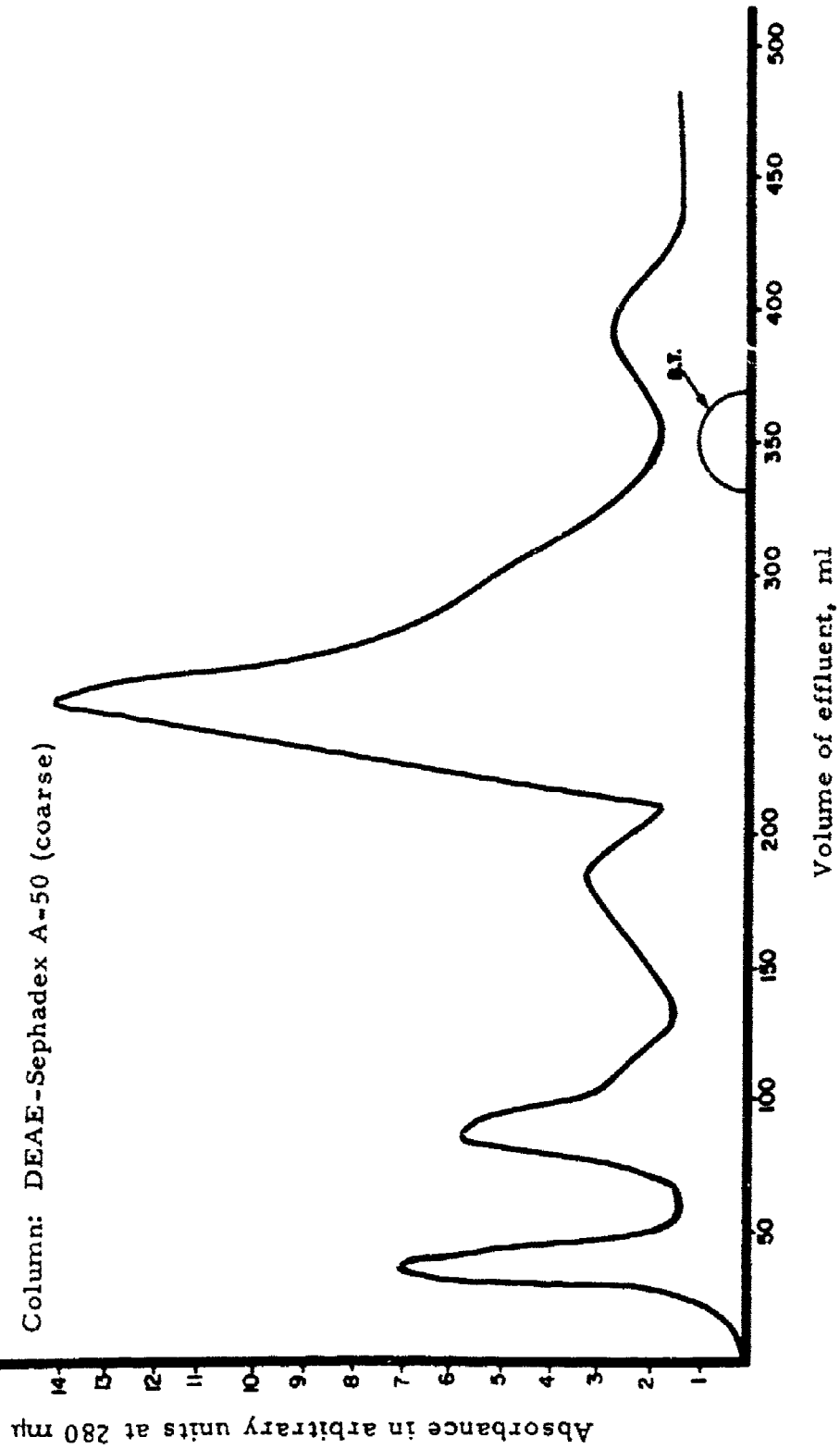
FIGURE 36

TYPICAL ELUTION PATTERN

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This procedure has made available a method by which the toxic fraction can be isolated and studied for particle molecular size. Attempts will also be made to isolate the toxic fraction from other tissues by this procedure.

DISCUSSION

Dr. Brooks (New York Medical College): What muscle did you use?

Dr. Sowinski: We used skeletal muscle from the femur.

Dr. Serrone (Albany Medical College): Skeletal muscle was used, the gastrocnemius. We tried to get as much muscle for him as we possibly could, so we made no distinction between aneural and neural ends. We just tried to get as much as possible.

Dr. Brooks: The diaphragm is equally suitable.

Dr. Sowinski: I intend to use this tissue to see if toxicity is present.

Dr. Zacks (The Pennsylvania College): It is easy to determine where the end plates are. There are many methods available, so that I think you could get a very clean aneural preparation for this type of study, using diaphragm or intercostal muscle or the gastrocnemius. If you cut it the right way, knowing where the end plates are, you have your preparation.

Dr. Sternberger (CRDL): This is a very promising approach, Dr. Sowinski. The material that you eluted could be either the original botulinum toxin or it could be the toxin after interaction with the tissues. Once the botulinum toxin has interacted with the tissues, it is not inactivated by antiserum. Could we distinguish its nature by the use of antiserum?

Dr. Sowinski: We could check that out.

Dr. Lamanna (Army Research Office): How do you know that this is the toxin that killed the animal?

Dr. Sowinski: It could be the excess toxin, that is true. There are a number of studies to be done. I would like to work with the smallest dose possible; in other words, have the rat die in 72 hr instead of 2 to 5 hr. Another experiment would be to label the toxin and inject as small a dose as possible that would still be detectable.

Dr. Lamanna: We know that if toxin is given iv, it stay⁻ in the blood a long time.

Dr. Sowinski: That is true. The way I feel about it, however, is that if you kill with as low a concentration as possible, you should be able to detect the active toxic molecule.

Dr. Lamanna: Has anyone done the experiment of taking muscle tissue, cleaning out the blood, adding antitoxin, and then separating out the muscle to see if there is in fact any selective uptake of the toxin?

Dr. Sowinski: I intend to isolate some of these fractions and see if there is any combination between the botulinum toxin and the fractions.

Dr. Coulston (Albany Medical College): Perhaps it would help if Dr. Sowinski pointed out what these peaks were, the fact that they were the various globulins and proteins of muscle and that no matter how we do it, the botulinum toxin always comes out at about 350, and whether we have a lot of excess in the blood or not is not so important at this stage of our work. We take the muscle and lyophilize it as throughly as we can and still keep it physiologic, but the important point is that whether we add the crystalline toxin to the muscle or whether we take it from an animal to which the toxin has been administered, it always comes through our column at that point. I think this is significant.

Dr. Sowinski: The fractions have not been identified; however, the second one is myoglobin. That is where the colored protein comes out, but the others I have not identified at all.

Dr. Sternberger: Is it not true, Dr. Lamanna, that the hemagglutinating principle has been reported by you as an entity different from neurotoxin? Also, according to your work, is it not true that the hemagglutinating principle possesses a wider, rather nonspecific range of adsorbability, being adsorbed, for example, by erythrocytes or by serum albumin? The people in Dr. Coulston's laboratory have succeeded in a very selective adsorption of the toxin in a highly specific fraction of muscle, and they have also detected this adsorption by neurotoxicity, not by hemagglutination assay. Would not these facts indicate a very important delineation of selectivity of localization of neurotoxin, important in our understanding of its mode of action?

Dr. Lamanna: Perhaps I can answer that question by asking another. What does anybody know about the relation of the hemagglutinin to the neurotoxin in the blood? Nobody knows anything. Whether or not the hemagglutinin in

fact is a separable substance is unknown, but there is also the good possibility that it is merely an activity expressed by a certain state of association of the toxin itself. So dragging in the hemagglutinin here only complicates the problem instead of helping.

Dr. Riesen: Have you any idea of the molecular size?

Dr. Sowinski: Experiments are planned to determine the molecular size.

FRACTIONATION AND FLUORESCEIN-LABELING
OF BOTULINUM TOXIN

Dr. Sumner I. Zacks
The Pennsylvania Hospital

The mission of our laboratory has been to prepare labeled botulinum toxin and to determine both the histological and the chemical sites of binding in experimental animals. This work was done in collaboration with Dr. Sheff, who, earlier this morning, described the preparation of the materials used in the morphological experiments that will be described now. First, some pictures of motor end plates will illustrate their structure.

The first illustration (figure 37A) demonstrates the relationships of motor nerves, blood vessels, and sensory innervation in a typical striated muscle. After methylene blue staining (figure 37B), the arborization of the terminal axons on the muscle fiber is clearly shown. This picture also shows that the end plates in many muscles are arranged in a more-or-less-regular pattern. A preparation of mouse intercostal muscle stained for AChE activity also shows this pattern (figure 37C). This regularity is very helpful in locating end plates in unstained muscle. Staining with silver or gold by various classical methods shows the terminal axons but little of the subneural component of the end plate (figure 37D).

In 1947, Couteaux greatly increased our knowledge of this structure by demonstrating that the subneural apparatus is composed of rodlets that extend into the underlying muscle surface. These rodlets can be seen in preparations stained supravitally with Janus green B (figure 38A) or by phase-contrast microscopy of 1μ plastic sections (figure 38B). Electron-microscopic studies of end plates have resolved many of the old controversies regarding their structure and have revealed the rodlets of Couteaux to be grooves in the muscle surface membrane (figure 38C).

(Figure 37A is from Adams, R. D. Diseases of Muscle. Hoeber Medical Division, Harper & Row, New York, New York. Figures 37B, 37C, 37D, 38A, and 38B are from Zacks, S. I. The Motor Endplate. W. B. Saunders, Philadelphia, Pennsylvania. 1964.)

Current Studies: Fluorescein-Labeled Botulinum Toxin.

In the initial phases of the work, chromatographic methods were used to separate and label various toxin fractions that were applied in preliminary in vitro binding experiments.

FIGURE 37

STRUCTURE OF MOTOR END PLATES STAINED
BY VARIOUS METHODS

A. Blood supply (ART) and innervation (MN) of striated muscle. Note
that majority of end plates are present in well-defined area in
center of muscle

B. Note axon (A), myelin sheath (M), and terminal axons (TA) lying on
muscle fiber (MF)   (X1,000)

C. Note rows of end plates (EP) on muscle fibers (X100)

D. Note nerve fibers (N), terminal axon, and end knobs (E) on muscle
fiber (F)   (X1,500)

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A

B

C
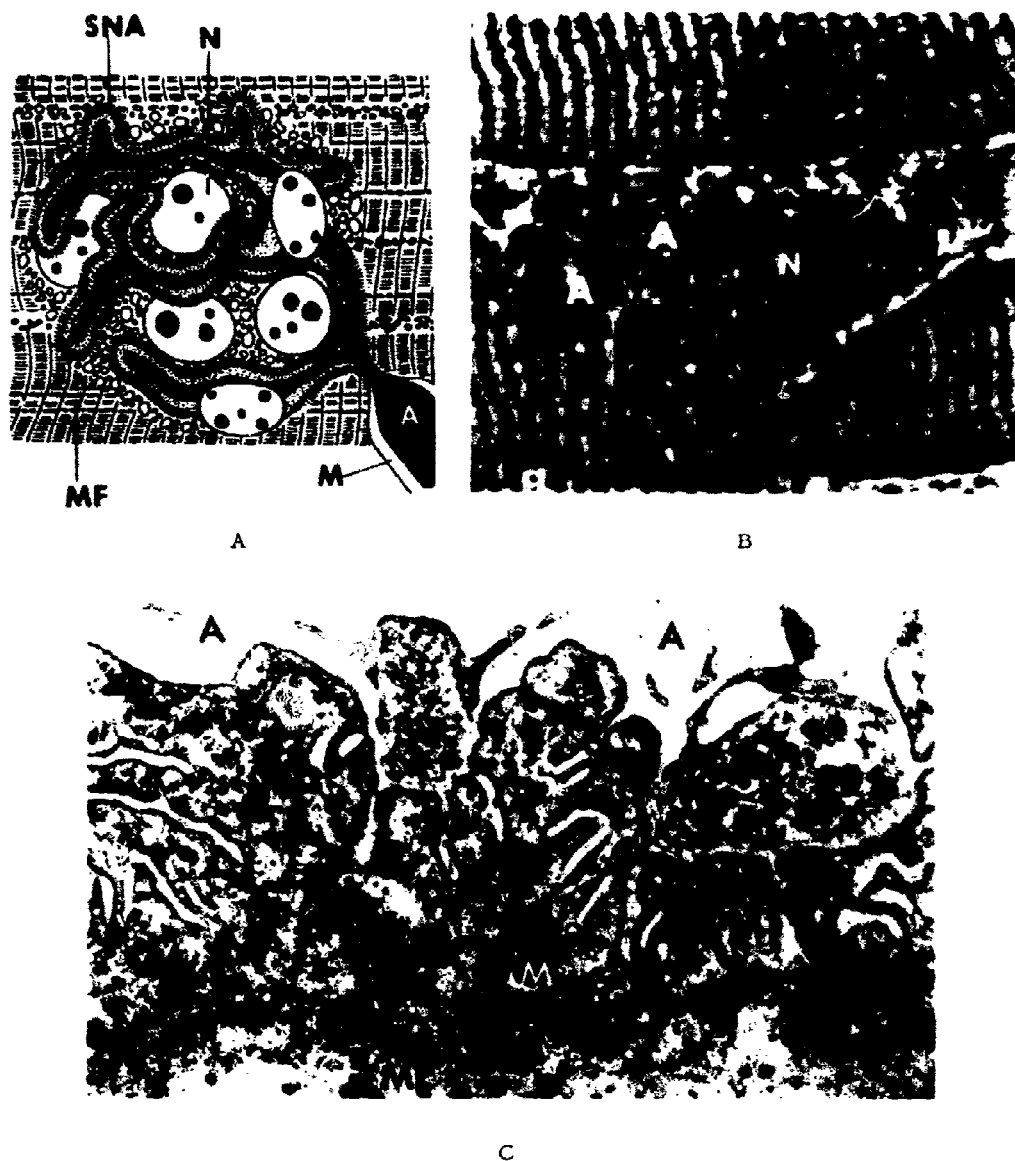
FIGURE 38

STRUCTURE OF MOTOR END PLATES SUPRAVITALLY
STAINED WITH JANUS GREEN B

A. Axon (A), myelin sheath (M), sole-plate nuclei (N), and subneural
apparatus (SNA) in relation to muscle fiber (MF) are illustrated

B. Note axons, nuclei (N), and subneural apparatus at arrows

C. Electron micrograph of motor end plate from dog intercostal
muscle. Note terminal axons (A), synaptic vesicles (SV),
synaptic clefts (SC), mitochondria (M), and muscle fibers
(X30,000)

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A typical in vitro experiment with labeled crystalline Type A toxin was done the following way:

Various organs from decapitated mice were frozen and sectioned at -30°C in a cryostat. The frozen sections were mounted on glass coverslips in wells and were incubated in a moist chamber for 30 min at 37°C with the labeled toxin (1 μg/ml) preparation. The sections were then washed 10 times in a buffered (pH 7.1) saline solution and were mounted in buffered (pH 7.1) glycerosaline on quartz slides. The controls for this experiment were normal tissues that were incubated with the tailings from the column. This solution contained the unbound rhodamine or fluorescein label. Rhodamine was used as well as fluorescein because it contrasts well with the normal bluish auto-fluorescence of many tissues.

Preliminary Results.

When unfractionated Type A toxin was applied to frozen sections in the in vitro situation described above, the brain and spinal cord showed slight diffuse (±) binding in both gray and white matter. Internal elastic membranes in meningeal arterioles were stained. The heart, spleen, liver, and lung all were unstained. In the small intestine, stomach, and colon, occasional small rhodamine-stained objects were observed in the lamina propria mucosa. The nature of these objects could not be definitely determined. It is possible that they represent sympathetic or parasympathetic cells. Diffuse binding of labeled Type A toxin occurred in skeletal muscle (figure 39A). Occasionally, irregular patches that were similar to end plates in size and shape were noted. Smooth muscle of the gut did not bind labeled toxin under these experimental conditions. Because this labeled toxin was a mixture of proteins, an attempt was made to increase the resolution of the method by use of a fractionated and dissociated labeled toxin, which Dr. Sheff has already described. In those experiments, fluorescein rather than rhodamine was used for labeling because of its greater photographic efficiency.

The labeled fractions of smaller molecular weight (70,000 to 150,000) showed differences in their binding characteristics when compared with the labeled crystalline Type A toxin. The dissociated toxin failed to bind to the brain and spinal cord but did stain the sarcolemma (figure 39B) and structures resembling end plates in size, shape, and location in the skeletal muscle. In transverse section, these structures appeared to be lenticulate or roughly curved around the periphery of the muscle fibers (figure 39C); in longitudinal section, they appeared to be irregular patches (figure 39D). Details of the subneural apparatus could not be resolved.
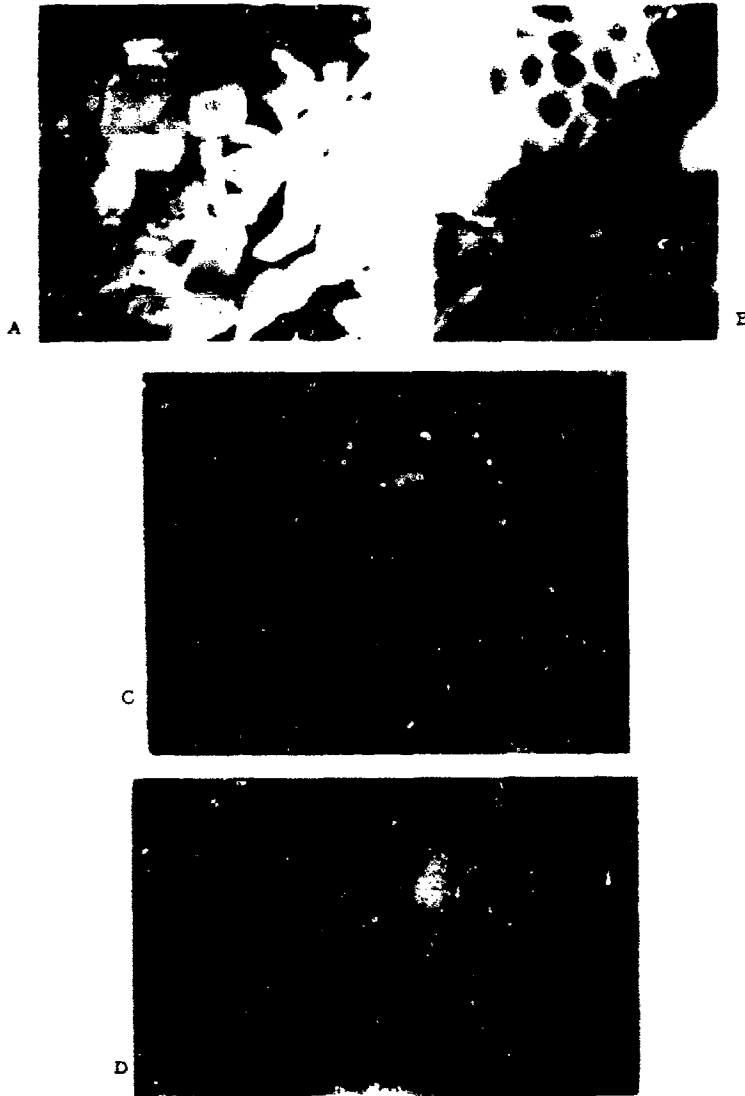
FIGURE 39

VARIOUS STRUCTURES STAINED WITH FLUORESCEIN-LABELED
BOTULINUM TOXIN

A. Mouse intercostal muscle stained with large fraction of toxin. Note
diffuse staining of fibers, including sarcolemma   (X100)

B. Mouse intercostal muscle stained with small fraction of toxin. Note
that myofibrils show only slight autofluorescence, whereas sarcolemma
is brightly stained   (X100)

C. Structure resembling end plate stained with toxin; transverse section
(X400)

D. Area resembling end plate stained with toxin; logitudinal section
(X400)

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Confirmation that these areas are end plates, by use of simultaneous-phase-contrast microscopy and other morphologic means will be necessary.

We especially want to do the obvious experiment with fluorescein-labeled botulinum antitoxin. Animals will be killed with toxin, their tissues will be removed, frozen, and sectioned, and then the sections will be treated with the labeled antitoxin to determine sites of binding. This type of experimentation has proved successful in studying the localization of tetanus toxin in a similar system.

This preliminary work points to some tentative conclusions. The labeled undissociated toxin binds to both muscle and brain in an apparently unselective way, whereas the dissociated toxin of lower molecular weight shows little affinity for brain, spinal cord, or myofibers but does stain the sarcolemma and the structures resembling end plates. Furthermore, the observed pattern of end-plate staining suggests that the subneural apparatus binds the labeled toxin. This, however, does not rule out possible binding in the presynaptic axon terminals, since the nerve fibers could not be resolved. This experiment will be repeated with labeled antitoxin.

Lead-Binding Experiments.

Savay and Csillik demonstrated that motor end plates selectively bind lead primarily in the membranes of the subneural apparatus. Since this reaction demonstrates a chemical property of these membranes that differs from the structurally similar (by electron microscopy) adjacent muscle surface membranes, this method was applied to the study of motor end plates that were acutely and chronically poisoned with botulinum toxin. The adaptation of this method for electron microscopy has yielded useful data on the embryogenesis of motor end plates (Kelly and Zacks).

Whole mounts of mouse hemidiaphragms were prepared from animals dying of acute intoxication or after 4 to 15 days of chronic intoxication. Whole mounts were selected for the initial study because the entire population of end plates could be observed and measured after application of the modified Csillik reaction. The opposite hemidiaphragm was fixed in 6% glutaraldehyde and prepared for electron microscopy.

The initial work that is being presented is limited to whole-mount material because of the trememdous amount of labor involved in surveying the large number of blocks prepared for electron microscopy. In a typical

experiment, there was no difference in size or staining characteristics of acutely poisoned end plates compared to normal ones, whereas significant changes were observed in the chronically poisoned animals. Two differences were noted in the chronically poisoned animals when compared with the controls: first, the end plates and associated muscle fibers were smaller and, second, there appeared to be less binding of lead by the subneural apparatus. Figure 40A shows a normal control end plate that can be compared with an acutely poisoned one (figure 40B) and a chronically poisoned one (figure 40C).

There is a direct relationship between muscle-fiber size and end-plate size and, therefore, an attempt has been made to closely match the size of the mice. Smaller muscle fibers (and end plates) in the chronically poisoned material might also be due to early muscle-fiber atrophy, possibly resulting from chronic intoxication. Table 24 shows the preliminary findings.

TABLE 24

LEAD STAINING OF MOUSE DIAPHRAGM MOTOR END PLATES
IN CHRONIC BOTULINUM POISONING

Preparation	Fiber width a/	End-plate size (max) b/	Intensity c/
	μ	μ	
Control	21.6 (22 ±3.6)	15.7 ±2.1	3.6
Chronic	16.7 (17 ±1.6)	13.3 ±3.8	1.8

a/ Fiber-width difference, P 0.001.

b/ End-plate-size difference, P 0.01.

c/ Arbitrary visual scale of 1 to 4[+].

At this point, the significance of this phenomenon cannot be assessed. If the work of Thesleff, Miledi, and others is correct and if there is a decrease in the amount of ACh secreted by the terminal axons in botulinum intoxication, the decreased affinity for lead may indicate a secondary phenomenon resulting from "chemical denervation." That it is not a primary effect is indicated by the fact that acutely intoxicated end plates do not show decreased lead binding.

FIGURE 40

NORMAL END PLATE AND END PLATES POISONED
WITH BOTULINUM TOXIN

A. Normal intercostal motor end plate. Note darkly stained
   subneural apparatus (X400)

B. Photomicrograph of intercostal motor end plate from
   acutely poisoned mouse (X400)

C. Photomicrograph of intercostal motor end plate from
   chronically poisoned mouse (X400)

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These experiments are being repeated under "double-blind" conditions to eliminate possible bias in the rating of staining intensity, and examination in the electron microscope is planned of control and acutely and chronically poisoned end plates stained with lead. In a few experiments in which the phrenic nerve was severed in rats, the pattern of end-plate lead binding seems to differ from those observed in chronic botulinum intoxication. There is an unfolding, or opening out, of the primary synaptic clefts and possibly an increase in lead binding in the early stages of surgical denervation. This observation suggests that we are dealing with a complex situation in the botulinum-intoxicated end plates.

The lead-staining technique is worth further study because, heretofore, there have been no morphologic characteristics with which to distinguish muscle surface membranes within the synaptic clefts from adjacent muscle surface membranes. That differences do exist has been proved by differences in lead binding and by numerous electrophysiological studies with recordings from microelectrodes.

The preliminary work has yielded whole and dissociated botulinum proteins labeled with fluorochromes, without significant loss of toxicity. Differences in in vitro binding have been shown by the various fractions in brain and in skeletal muscle. The dissociated toxin of lower molecular weight demonstrates more selective binding, particularly in the sarcolemma and in structures tentatively identified as motor end plates. The morphologic evidence suggests that the subneural apparatus is stained, although staining of the terminal axons cannot be excluded. Experiments with labeled anti-toxin in acutely and chronically intoxicated animals are planned. The labeled toxin preparations will also be used to trace absorption in the gastrointestinal tract.

DISCUSSION

Mr. Fleisher (CRDL): I would like to ask Dr. Zacks whether it might be possible, by the use of serial sections, to have the morphology by conventional procedures on one section followed by another in direct sequence with the fluorescent labels, so that there might be some verification of the location of the fluorescence.

Dr. Zacks: That is the sort of thing we have in mind. The reason we call them end plates at all is their morphology and by exclusion of other structures in the area. If the intercostal muscle block is taken between two ribs, end plates are found in a narrow band in the center of the fibers. The appearance of these structures in transverse sections is consistent with end plates cut on edge, as is their differing appearance when the muscle is cut longitudinally. Unless they are precipitation artifacts (and it seems that they are too regular for that), they could only be confused with medium-sized nerve fibers or vessels. I think the illustrations rule out those possibilities. We intend to use a combined phase contrast—UV technique, if we can acquire the apparatus.

Dr. Petty (Maryland Medical-Legal Foundation, Inc.): Does this fluorescent technique of yours work just with teased muscle preparations?

Dr. Zacks: We have not used teased preparations, but I don't see why there should be any difference other than one that might be controlled; namely freezing. I think it would be a useful technique to try.

Dr. Petty: The reason I asked is, it would seem that if you wish to prove the presence of the end plate in the preparation, all you need to do is to tease apart several of the fibers, and you should be able to identify that very nicely as the end-plate area by your fluorescent technique. This would be one approach to it.

Dr. Brooks (New York Medical College): It occurs to me that the hemicholinium method of paralysis referred to somewhat earlier in the discussion might be worth looking at because it prevents the methylation of the choline further back in the chain of events and, therefore, the accumulation and production of ACh, and it is a presynaptic event.

Dr. Zacks: I think that is a good suggestion, and we would like to do it, although it may be a little beyond the immediate confines of our contractual responsibility.

Mr. Fleisher: I wonder if it might be possible to use the Savay-Csillik technique in vivo in an animal, perhaps with one leg chronically denervated; the other, of course, with nerve and neuromuscular junctions intact and then injecting the labeled toxin, and then, again, looking for differences in the appearance of the fluorescent- (or otherwise) labeled molecule at the neuromuscular site?

Dr. Zacks: One problem that we have had some experience with is that if you start with the labeled toxin and try to find it after in vivo injection, it is very difficult to get enough toxin into the animals to have enough fluorescent molecules to see in UV. For example, a mouse dies after injection of 9 μg of tetanus toxin, the minimum saturation dose. This amount of material is much too small to find by direct UV microscopy. If you inject very large amounts of a highly labeled toxin, you can succeed under some circumstances. However, when you consider the low lethal dose for botulinum toxin, the problem seems a hundredfold greater. The thing you can do is use labeled antitoxin or a sandwich of toxin-antitoxin and anti-antitoxin with label. We hope to do that as soon as we get the reagents.

Dr. King (IITRI): Do you also plan to label your small unit with fluorescein?

Dr. Zacks: We would very much like to do that if we could, but so far we are certain only of the 50,000 to 170,000 unit.

Dr. King: I would like to make a comment about Dr. Sheff's presentation. You have been talking about the time to death of the smaller preparations. I didn't point out this morning that most of the toxicities we studied were the 24-hr type, but when we had the separation of the pooled fractions from the electrophoretic material (the fraction that was completely small), all the deaths occurred within 24 hr. With the large fraction, we had deaths up to 72 hr.

ATROPHY IN EMBRYONIC CHICK MUSCLES AFTER MASSIVE DOSES OF BOTULINUM TOXIN

Dr. Daniel Drachman
Tufts University School of Medicine

I appreciate the opportunity, not only to hear what has been discussed, but also to present some of my ideas and see what comments they will elicit.

When a motor nerve is severed, the muscle it supplied is immediately paralyzed, since the phasic impulses are no longer conducted from the nerve cell body to the muscle fiber. More prolonged denervation results in a loss of the nerve's trophic effect on the muscle as well. Although the trophic action of the nerve is difficult to define precisely, it maintains the physiological and anatomical integrity of the muscle. Loss of the trophic action results in various phenomena in the muscle, including the following:

1. Atrophy, and ultimately degeneration of the muscle

2. Spread of sensitivity to ACh from the end-plate region, to include the greater part of the muscle membrane

3. Spontaneous contractions of individual muscle fibers (fibrillation)

4. The ability of the muscle to accept innervation by an implanted nerve

5. A vast number of metabolic changes that are beyond the scope of this presentation.

Botulinum toxin interferes with transmission of the nerve's phasic activity by preventing neural ACh release. It may be asked, however, whether botulinum toxin will abolish the trophic activity of the nerve as well, if it is applied for a sufficiently prolonged period. In other words, does it produce a "pharmacological denervation of muscle"?

Previous work by Thesleff's group in Sweden has shown that botulinum toxin can, indeed, interrupt several of the nerve's trophic actions. Toxin-treated muscle develops an enlargement of the ACh-sensitive membrane area. Spontaneous fibrillations appear in botulinized muscle. Recently, Hoffmann and Thesleff have shown that botulinum-poisoned muscle will accept reinnervation by an implanted nerve in the same manner it will accept reinnervation if the original nerve has been severed.

The foregoing evidence clearly indicates that botulinum toxin interferes with the trophic influence of the nerve on certain physiological properties of muscle. During the past 2 yr, I have conducted a series of studies to determine whether treatment with botulinum toxin produces anatomical atrophy of muscle as well. First, it was necessary to find an animal that would survive large doses of the toxin. The lethal effect of botulinum toxin is due to its paralysis of the muscles used for respiration; accordingly, an animal that does not depend on skeletal-muscle activity for respiration might be suitable. Such an animal is the chick embryo. During embryonic life, respiration is effected by passive diffusion of gases across the chorioallantoic membrane. Because the chick embryo readily survives very large doses of botulinum toxin, I have used it as the experimental animal throughout the present study. (Let me assure you that I am aware that it is a chick, and that it is an embryo.) Undoubtedly, someone will point out and object to the fact that since I am using a chick embryo, the conclusions reached cannot apply to mammals, and certainly not to adult mammals. I am, however, considering the chick embryo as an in vivo model of a neuromuscular system.

In all of the present experiments, botulinum toxin was injected iv into the chorioallantoic circulation using a technique that we have described previously.* The embryonated egg is candled to locate a large chorioallantoic venous bifurcation. The air sac is punctured, and a window is removed from the shell and shell membrane overlying the vein. After the chorioallantoic membrane drops, a specially made polyethylene microcatheter is introduced into the vein, under a dissecting microscope. The injection is made through the catheter by means of a micrometer-driven syringe.

Injections were begun after either 7 or 12 days of incubation and repeated at 3-day intervals thereafter. The embryos were sacrificed on the 19th day. Each injection contained 39 to 54 µg of Type A botulinum toxin diluted in Ringer's solution to a volume of 0.1 ml. This amount represents approximately 10,000 lethal doses for hatched chicks. The embryos first injected after 7 days, therefore, received a total of 40,000 lethal doses in the course of the experiment.

The youngest embryos used in the present experiments were 7 days old. At this time, anatomical contact between nerve and muscle has already been made and can be visualized using silver stains and the light microscope. We are, therefore, dealing with innervated muscles throughout this study and have avoided consideration of the more primitive embryonic

* Drachman, D., and Coulombre, A. J. Science 138, 144 (1962).

muscle prior to innervation. By 12 to 14 days of age, the neuromuscular contact is more mature in the normal chick embryo. At this stage of development, motor end plates containing discrete accumulations of cholinesterase are seen, using histochemical techniques. By the 19th day of incubation, the muscle and myoneural junction of the normal chick embryo closely resemble those of the adult chicken.

Treatment with botulinum toxin results in marked atrophy, degeneration, and fatty replacement of muscle in the chick embryo. Figure 41 shows a normal chick at 19 days of incubation, with the skin removed to demonstrate the skeletal muscle. The 19-day-old embryo in figure 42 received four doses of botulinum toxin. There is very little grossly visible muscle remaining. The white substance infiltrating and replacing the muscles is fat. The joints of the experimental embryos are ankylosed. In a separate series of studies, I have found that skeletal-muscle contractions are essential for the primary development and maintenance of embryonic joints.

Figure 43 is a cross section through the quadriceps muscle of a normal 19-day-old chick embryo. Figure 44 was taken at the same magnification, through the same region of the thigh, in a 19-day-old embryo that was treated with botulinum toxin. Virtually all of the muscle has been replaced by fat. The posterior thigh again shows massive fatty replacement of muscle. The sciatic nerve and a few remaining muscle fibers are also present. At higher power (figure 45),these muscle fibers are seen to be myotubes; namely, each fiber has a central canal partially filled by nuclei. Myotubes are relatively immature muscle fibers, and they constitute a very small proportion of the muscles of normal embryos older than 14 to 15 days. In contrast, normal muscle fibers at 19 days (figure 46) do not contain central canals. The nuclei are found just beneath the sarcolemmal membrane.

Figure 47 is a longitudinal section through the entire thickness of the anterior leg muscle. There has been massive loss of skeletal-muscle bulk. The remaining fibers show a great relative increase of sarcolemmal nuclei. Many fibers show flagrant evidence of degenerative changes, with loss of striations, swelling, and infiltration by histocytes.

These experiments show that prolonged treatment with botulinum toxin produces a dramatic change in skeletal muscle, consisting of atrophy, fatty replacement, and degeneration, with immaturity of the persisting muscle fibers.

FIGURE 41

NORMAL 19-DAY CHICK EMBRYO, WITH SKIN REMOVED

FIGURE 42

A 19-DAY CHICK EMBRYO TREATED WITH 10,000 LETHAL DOSES OF
TYPE A BOTULINUM TOXIN ON DAYS 7, 10, 13, AND 16

(Skin has been removed; note marked reduction in skeletal muscle, and
replacement by whitish fat; joints are ankylosed)

155
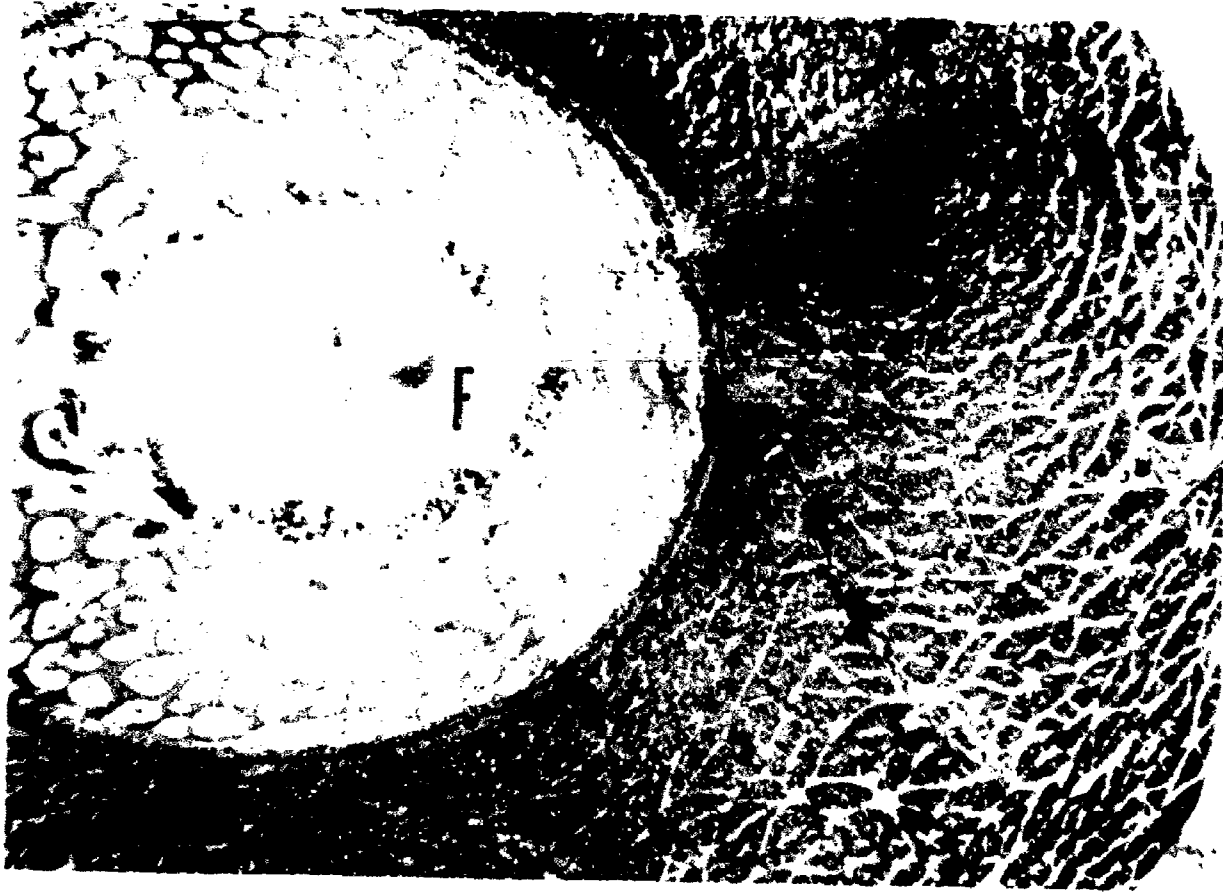
FIGURE 43

CROSS SECTION THROUGH FEMUR AND QUADRICEPS MUSCLES OF NORMAL
19-DAY CHICK EMBRYO

(Femur is labeled F, X70)

FIGURE 44

CROSS SECTION THROUGH IDENTICAL AREA AS IN FIGURE 43 OF
19-DAY EMBRYO THAT HAD RECEIVED FOUR DOSES OF
BOTULINUM TOXIN

(Note virtually complete replacement of muscle by fat; femur is labeled F;
arrow indicates remaining muscle fibers; X70)
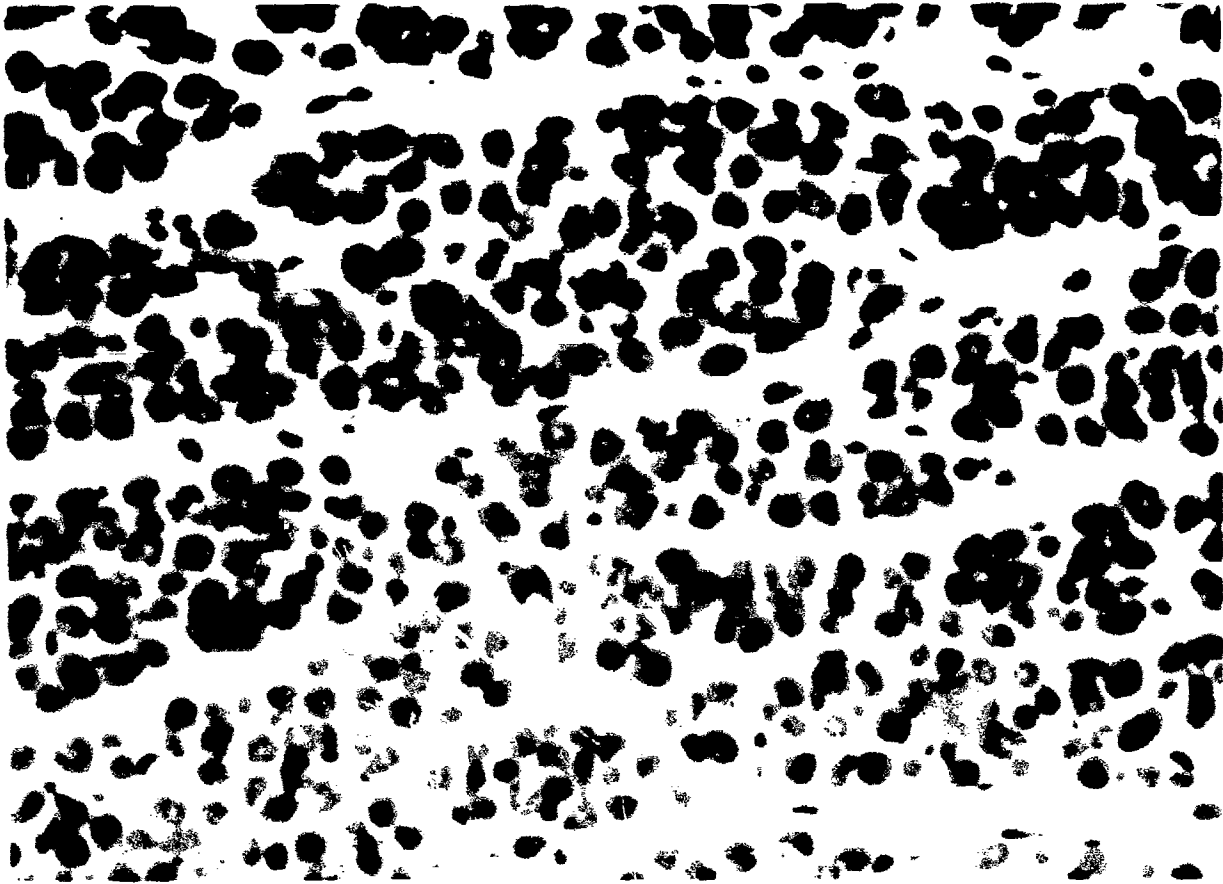
FIGURE 45

HIGH-POWER VIEW (APPROXIMATELY X780) OF REMAINING MUSCLE
FIBERS IN 19-DAY, BOTULINUM-TREATED EMBRYO

[Note myotubal (doughnut) appearance in cross section]
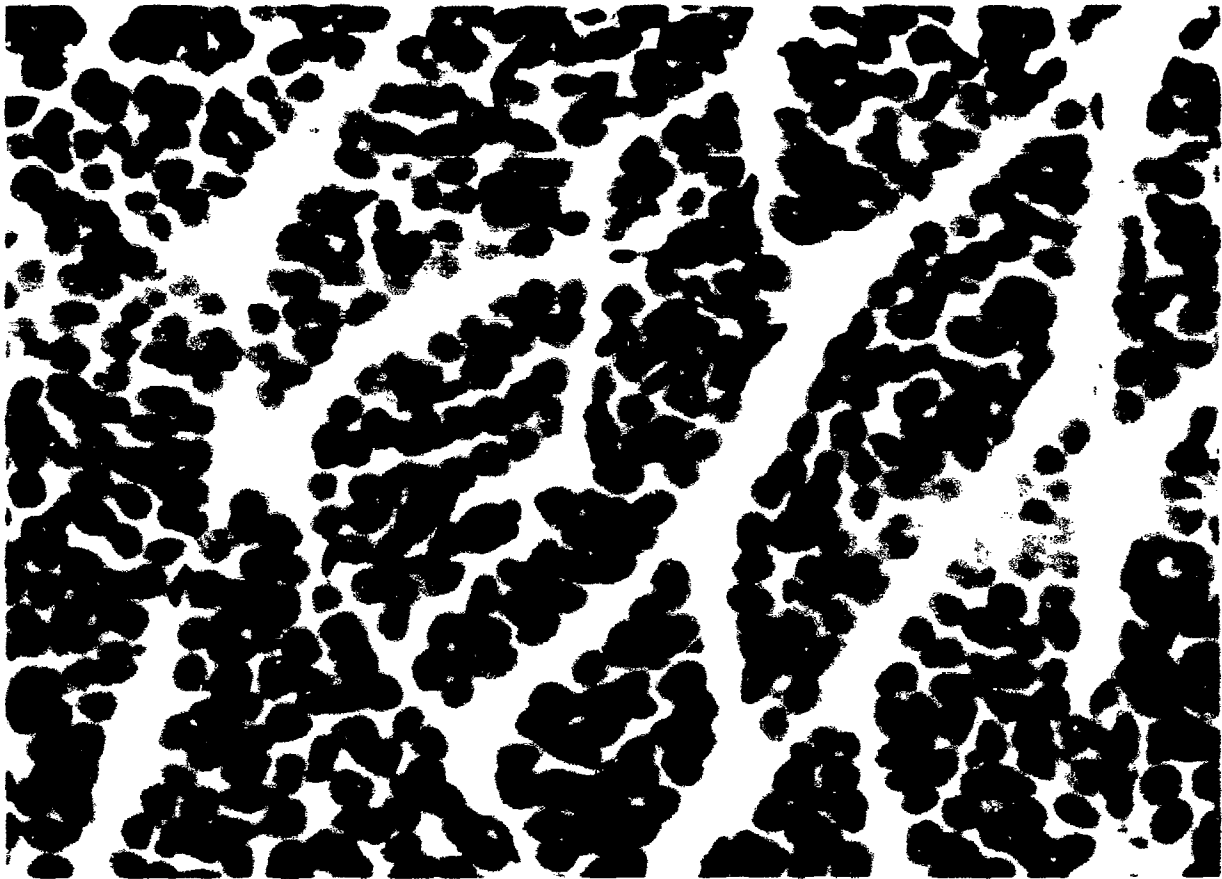
FIGURE 46

CROSS SECTION THROUGH MUSCLE FIBERS OF
NORMAL 19-DAY CHICK EMBRYO

(Note peripheral location of sarcolemmal nuclei and absence of
central canal; approximately X780)

FIGURE 47

LONGITUDINAL SECTION THROUGH ANTERIOR LEG MUSCLE OF 19-DAY
CHICK EMBRYO TREATED WITH FOUR DOSES OF BOTULINUM TOXIN

(Tibia is labeled T; note marked reduction in number of muscle fibers
and replacement by fat, labeled F; muscle fibers show degeneration
and cellular infiltration)

Several questions arose as to the mechanism and significance of this phenomenon, which have led to the following analysis:

1. Is the effect observed in the present experiments attributable to the toxin itself or to some other feature of the experimental procedure?

    Control injections of (a) Ringer's solution and (b) toxin and antitoxin had no effect on skeletal muscle. In fact, the "normal" embryonic muscle shown in figure 43 is from one of the chicks treated with toxin and antitoxin. The pharmacological action of the toxin itself is responsible for the present findings.

2. Does botulinum toxin cause "general retardation of growth and development" sufficient to account for the muscle findings?

    a. The treated embryos were normally mature for their age, although somewhat smaller and lighter in weight than the controls.

    b. In untreated embryos, the growth rates of liver and lower extremities are parallel between the 7th and 19th days of incubation. Therefore, an agent that causes general retardation of growth during this period would be expected to interfere with the growth of the liver and the lower extremities to a comparable extent. The botulinum-treated embryos showed a reduction in lower-limb muscle weight of 76.4% to 85% (as compared with the normal), whereas the liver weight was reduced by less than 18%. These findings cannot be accounted for by a "generally retarding" process.

3. Does botulinum toxin exert a direct effect on striated muscle? Cardiac muscle, like skeletal muscle, is striated. It does not depend on innervation, however, for maintenance of its integrity. The heart muscle of the botulinum-treated chick embryos was histologically and functionally normal although its weight was slightly reduced. This negative finding is suggestive evidence that botulinum toxin does not exert a direct toxic effect on striated muscle.

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4.   Is the histological appearance of the botulinum-poisoned
       skeletal muscle consistent with the picture of denervation
       in the chick embryo?

       Previous experiments of Eastlick, Hamburger, and other
embryologists have shown that noninnervated limbs can be developed by graft-
ing limb buds to parts of the embryo or its membranes remote from the
spinal cord. These noninnervated limbs showed degeneration, atrophy, and
striking fatty replacement of muscle fibers, entirely consistent with the
findings in the present experiment. I have examined this question further by
destroying the lumbosacral spinal cords of the 6-day-old embryos and
observing the effects on the limbs. By 19 days, the findings are similar to
those in the botulinum-treated limbs, although more advanced. Figure 48
shows the lower limbs of an embryo after spinal-cord extirpation. The marked
atrophy of muscle and fatty replacement are obvious. Further histological
examination of these limbs has confirmed the similarity between the effects of
surgical denervation and those of prolonged botulinum treatment on skeletal
muscle in the chick embryo.

5.   Does botulinum toxin produce muscle atrophy by primarily
       damaging motor neurons?

       Histological examination shows that motor neurons in treated
embryos are structurally intact, whereas the muscles they innervate have
become severely atrophic.

       Intramuscular nerve fibers are seen on silver staining in the
midst of very atrophic muscle. A normal complement of anterior horn cells
is present in the chick embryos treated from the 12th through the 19th days.
We may conclude that botulinum toxin produces muscle atrophy by means of
its pharmacological action, rather than by direct primary neural damage.

       There is a definite loss of anterior horn cells in the spinal cords
of embryos treated from the 7th through the 19th days of incubation. This
phenomenon may be similar to the loss of anterior horn cells seen in young
chick embryos after amputation of a limb ("loss of the peripheral field of
innervation"). Its mechanism remains to be determined.

6.   Is the disuse of muscle caused by botulinum paralysis
       responsible for the atrophic and degenerative changes?

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FIGURE 48

LOWER LIMB OF 19-DAY CHICK EMBRYO AFTER SURGICAL REMOVAL
OF LUMBOSACRAL SPINAL CORD ON SIXTH DAY

(Note absence of muscle tissue; femur and tibia are clearly seen through
overlying fat; joints are ankylosed)

This question cannot be answered fully and satisfactorily by any available experimental method. Unfortunately, we have no way of producing complete muscular disuse without denervating surgically or pharmacologically. Tenotomy, however, affords a fairly good approximation of disuse, by depriving the muscle of its external load. The resulting disuse, of course, is only relative, since the muscle can still contract and perform work against its own viscosity. At any rate, I have tenotomized the quadriceps of 7-day-old chick embryos by performing through-the-knee amputations. These operations resulted in a mild degree of atrophy and no degeneration of the quadriceps muscle. Although not conclusive, this experiment suggests that disuse is not the mechanism by which botulinum toxin produces skeletal-muscle atrophy in the chick embryo.

7. Can the skeletal-muscle atrophy be attributed to interference with the function of higher neural centers by botulinum toxin?

Recently, Tyler and Polley and coworkers have demonstrated that certain physiological changes occur in the CNS as a result of botulinum poisoning. In order to rule out the rather remote possibility that interference with higher nervous function might be responsible for the muscular atrophy, I decapitated chick embryos at 7 days and incubated them until the 19th day. These headless specimens survive well and have a normal amount of intact, functioning skeletal muscle.

From these studies, we may conclude that botulinum toxin, by virtue of its specific pharmacological action, reproduces the morphological changes of denervation in the chick embryo. It is tempting to attribute this effect to suppression of neural ACh release, and to speculate that ACh may be the "trophic factor" elaborated by nerves. We cannot, however, exclude the possibility that botulinum toxin, besides inhibiting ACh release, may prevent the passage of some other, unknown, trophic substance from motor nerve to muscle.

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DISCUSSION

Dr. Zacks (The Pennsylvania Hospital): I congratulate Dr. Drachman on this study, that,I think if Dr. Singer's work on regeneration is any model, we'll find him working on for the next 10 yr. , It is obviously a very complicated system,and I should like to ask a question. One control not mentioned was the injection of inactivated botulinum toxin, boiled or otherwise denatured, and injected to see what happens. Another question is about the observation that in the pictures there is very little fibrosis that one sees in degenerating and replacing muscle. It may be that this is an instance where this does not occur in embryonic material, but it is striking to see the muscle entirely replaced by fat with very little fibrocytic reaction. This is interesting. The point about using cardiac muscle as a standard for striated muscle is hazardous, I think, for we found in work with tetanus toxin that where you can show very marked binding to skeletal muscle of the periphery; it won't bind to the heart muscle. We know that there are differences ultrastructurally and, certainly, the myosin seems to be different, not to mention the mitochondria and other components. One thing I am curious about is what the end plates look like in muscle that is not completely altered, as in the 19th-day embryonic muscle. There are many minor points that come up, and I think that in the future Dr. Drachman will contribute much more with this very nice system.

Dr. Drachman: Thank you, Dr. Zacks. Concerning inactivation of the toxin, I had planned to use two methods: (1) the addition of antitoxin; (2) heat denaturation. The results of the antitoxin inactivation were so successful that I did not feel that the heated toxin control would be necessary.

As to the question of fibrosis versus fatty change in denervated muscle, I refer again to figure 48,which shows the massive fatty replacement in surgically denervated muscle. Likewise, Eastlick and Hamburger have commented on the striking picture of fatty replacement of denervated embryonic muscle. Although the fatty replacement is far more impressive, fibrosis does occur in denervated or botulinum-treated embryonic muscle. The fibroblasts, which invade the area, are spindle-shaped and appear to be very immature.

Concerning the question about end-plate development: Using histochemical techniques for the demonstration of ChE, I have found that very few end plates may be seen in the botulinum-treated embryos. One cannot, however, be sure whether or not the loss of end plates is due to disruption of the muscle fibers themselves, as described above. Quantitative studies of ChE may not prove helpful either. Goodwin and Sizer at M.I.T. attempted

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to measure the ChE content of denervated skeletal muscle in chick embryos. They found that the variability in the muscle ChE level among chick embryos was too great to establish whether denervation suppressed the formation of ChE. Furthermore, the relatively large amount of myosin ChE provides too much "background noise, " which obscures the relatively small changes that might be expected at the end plates as a result of denervation.

Dr. Zacks: The immunological mechanism is not developed in the chick embryo, but you should be able to find that complex over the botulinum and the protein present, would you not? I mean, it would not be a side effect.

Dr. Drachman: The toxin itself?

Dr. Zacks: Toxin plus—there is a conflict there between the proteins.

Dr. Drachman: In other words, one ought to be able to administer toxin followed by antitoxin and then locate it. Is that what you are saying?

Dr. Zacks: Yes.

Dr. Drachman: That sounds reasonable; however, the chick embryo does develop immunological competence during embryonic life.

Dr. Sternberger (CRDL): On the 14th day, the immunological mechanism is sufficiently developed to see what a very small amount of toxin-antitoxin accomplishes.

Dr. Lamanna (Army Research Office): There is an observation that Lawrence Layton and I made to support the contention that there are no nonspecific effects on heart muscle. In tissue culture, the natural beat of isolated pieces of the chick heart (obviously unconnected to any nervous system) is not affected by addition of botulinum toxin.

Dr. Drachman: This certainly goes along with these in vivo observations that huge doses of toxin do not interrupt the normal cardiac output.

Dr. Sternberger: These chick-heart cells beating in tissue culture are without any innervation whatsoever, so you would not expect them to be affected in any way by botulinum toxin, would you?

Dr. Drachman: That is right. The only reason for using cardiac muscle is that it furnishes an example of striated muscle that is not dependent on the trophic effect of innervation.

Mr. Fleisher (CRDL): In your observations you alluded to the possibility of some secondary factor developing as a result of botulinum poisoning of the embryo, and this is a fascinating but very intangible thing to pin down. I wonder, if one took sibling embryos and poisoned one but not the other as some type of control, whether at a time when your observations indicate a full development of the poisoning and the pathogenesis, it might be possible to prepare from parts of the poisoned embryo some type of extract and inject this into the appropriate control and see what might develop as a means of, perhaps, verifying the existence of a secondary factor.

Dr. Drachman: The question of the "trophic factor" is a very difficult one. From a speculative point of view, I would agree with Thesleff that it is the local, properly timed release of ACh at the neuromuscular junctions (which produces the miniature end-plate potentials), that is responsible for the preservation of muscle. I do not believe that ACh acts as a hormone. Nor do I agree with Guttmann that the nerve releases some protein substance, which exerts a trophic influence on muscle.

There are two published experiments that indicate that conduction of impulses along a motor nerve is not necessary for maintenance of the trophic effect. Denny-Brown and Brenner produced reversible local nerve block by traumatizing the nerve along its course, and Guttmann and Zak infused local anesthetics along motor-nerve trunks. Each of these procedures interrupted propagation of the action potentials down the nerve, but did not interfere with the spontaneous quantal release of ACh from the nerve endings. In these experiments, there was no loss of the trophic activity of the nerve.

Dr. Zacks: Just a comment; is it possible that if you are giving the toxin from the 7th to the 12th days, you are interfering with the anlagen of the skeleton? In other words, I am now speaking as a teratologist, which I am not, but that is the idea. Two things then; since you use the toxin at a point when most teratologists would give it, between the 7th and 12th days, do you see, for instance, cleft palate and things of this sort or are you implying that is is a teratogen? I am trying to bring you back to the embryology.

Dr. Drachman: Ordinarily, teratogens exert a more profound effect when they are given within the first few days of embryonic development. By the 7th, and certainly by the 12th days of embryonic life, the skeletal structures are fairly well developed. Therefore, I would not expect that the effect of

botulinum toxin in these experiments is a gross teratogenic one. Apart from the muscular atrophy that I have described today, the only abnormality found in the botulinum-treated embryos is a condition known as arthrogryposis. This is a congenital disease, akin to clubfoot, that consists of ankylosis of more than one joint. This disorder is due to lack of movement of the embryo that is required for normal development and maintenance of joints. I have previously reported, in a series of papers, that paralysis caused by curare, succinylcholine, decamethonium, botulinum toxin, spinal-cord destruction, or by rubella destroying the anterior horn cells of the embryo will result invariably in such joint deformities.

Dr. Drachman: I have read a report of a human case of arthrogryposis in which the pregnant mother was intimately exposed to German measles and evidently developed an inapparent infection. Presumably, the embryo was infected at the same time. He was born with arthrogryposis and an impressive diminution of the number of anterior horn cells, some of which could be seen in various stages of destruction.

Dr. Sternberger: I think that large quantities of German-measles virus have been recovered for at least a year from the urine of German-measles infants, indicating thereby a very large and persistent dissemination of the virus.

You are talking about chick embryos, and it is interesting that these animals do survive and that they do not need a diaphragm for respiration. Do you think you can stretch the analogy and perhaps use more adult animals and support muscle movements by other means, as well as the respiration, and get recovery from botulism that way?

Dr. Drachman: There is no real objection to using botulinum toxin in adult animals, providing one supports their vital functions. Botulinum, however, may possibly cause disturbances of autonomic function because of interference with ACh release. This, and perhaps intravascular agglutination, may prove fatal in spite of assisted respiration. Perhaps Dr. Tyler can comment on that.

Dr. Zacks: Certainly I would say that with present-day medicine, respiration is not a problem for 4 or 5 wk. The problems of botulism patients are often much smaller, not paralysis, but the secondary complications that paralysis leads to: the chest infections, the pneumonias, the cardiac irritability, and so on that usually get you into trouble at the bedside.

Dr. Wills (CRDL): This is going to be discussed tomorrow, so I would say that we should not get into it very deeply now.

Dr. Zacks: Just one point. Shouldn't you really test your hypothesis by blocking the synthesis of ACh rather than using something like curare, which doesn't have anything to do with the real difficulty?

Dr. Drachman: You are referring to the use of hemicholinium?

Dr. Zacks: No, I am referring to triethylcholine. Wouldn't this be a more appropriate way?

Dr. Drachman: No, I didn't mean to bring up the curare experiment as further evidence for this denervation but only for the arthrogryposis. That is quite different. You are quite right; it is something that needs to be done. Yes.

SESSION III

29 June 1965

Chairman: Dr. Charles C. Hassett
Assistant Chief, Physiology Division, CRDL

PHOTODYNAMIC TREATMENT OF BOTULINUM POISONING

Mr. Leo Feinsilver, Lt J. G. E. Vestweber, and Mr. Frank J. Vocci
Toxicology Division
CRDL

An appropriate subtitle of this section would be Photodynamic Detoxification of Botulinum Toxin in Mammalian Blood.

In considering new approaches to the in vivo detoxification of botulinum toxin, the concept of photodynamic action appeared challenging. This concept is founded upon many researches that have shown that cell cultures, enzymes, unicellular organisms, and so forth can be inactivated or even destroyed by being subjected to the action of photosensitizing compounds, such as certain dyes, in the presence of light and oxygen. For example, Bolande[1] has described the cytotoxic action of eosin upon living-cell cultures. Weil and coworkers[2] have shown that the toxicity of botulinum toxin can be rapidly and almost completely abolished by adding methylene blue (MB) in low concentrations and subjecting the mixture to the action of visible light and air. The esterase activity of chymotrypsin can be inhibited or completely destroyed by the action of photodynamic dyes. Many other similar effects have been reported.

Different mechanisms of photodynamic action have been proposed by several investigators as special cases of photosensitized oxidation. Some of these schemes are shown below.

In Blum's[3] generalized scheme, the photosensitive dye is activated (or excited) by capture of a photon of light energy. The activated dye, in intimate contact with an oxidizable substrate, transfers its energy to this substrate, which in turn reacts with oxygen, resulting in an oxidized or inactivated substrate.

In Kautsky's[4] scheme, the activated dye transfers its energy to a molecule of oxygen, resulting in an activated oxygen molecule capable of oxidizing a nearby sensitive substrate.

In the scheme of Heinmets,[5] which was applied to the inactivation of certain viruses, the dye first combines at critical sites in the virus particle to yield a dye-virus complex D·X. This complex is excited by the absorption of a quantum of light energy. The excited complex then combines with oxygen, resulting in a loss of infectivity. The complex containing the inactivated virus remains in equilibrium with the unbound dye.

Blum

$$D + h\nu \longrightarrow D*$$

$$D* + X \longrightarrow X* + D$$

$$X* + O_2 \longrightarrow X_{ox}$$

Kautsky

$$D + h\nu \longrightarrow D*$$

$$D* + O_2 \longrightarrow D + O_2*$$

$$O_2* + X \longrightarrow X_{ox}$$

Heinmets

$$D^+X^- \longrightarrow D \cdot X$$

$$D \cdot X \longrightarrow (D \cdot X)*$$

$$(D \cdot X)* \xrightarrow{O_2} D \cdot X^o$$

$$D \cdot X^o \longrightarrow D + X^o$$
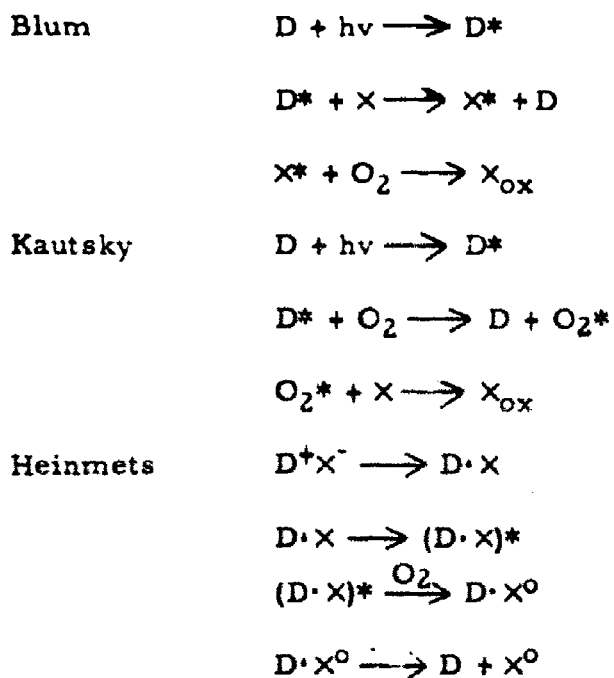
where

D or D^+ = photosensitive dye

$D*$ = activated dye

$h\nu$ = quantum of energy

X = oxidizable substrate

$X*$ = activated substrate

X_{ox} = oxidized substrate

O_2* = activated oxygen molecule

$D \cdot X$ = dye-virus complex

X^- = infective virus substrate

X^o = inactivated virus

$(D \cdot X)*$ = activated dye-virus complex

$D \cdot X^o$ = dye-inactivated virus complex

At the last American Chemical Society meeting in Chicago, Dr. Foote, Dr. Wexler, and Dr. Ando[6] of the University of California reported that the mechanism of reaction of olefins with singlet oxygen (molecular oxygen in an excited singlet state) closely resembles that of the dye-sensitized photooxidation of olefins. They compared the stereochemistry of the reaction of limonene with singlet oxygen to that of its photochemical oxidation and showed that the two reactions gave remarkably similar product distributions. This led to the conclusion that identical intermediates are formed in the two reaction modes. These findings led substantial support to Kautsky's mechanism of photodynamic action.

Bellin,[7] at the Polytechnic Institute of Brooklyn, showed that the ability of dyes to photosensitize the autooxidation of an organochemical substrate is paralleled by their ability or inability to sensitize the inactivation of the DNA transforming principle; that is, a biological substrate.

On the strength of the foregoing observations, a program was initiated in our laboratory to study the photodynamic action of several dyes, particularly MB, upon botulinum toxin, especially in the presence of mammalian blood.

An outline of the experiments that have been performed on the photodynamic action of dyes upon botulinum toxin is presented below.

I. Model in vitro systems using mammalian blood

    A. Static systems

    B. Circulatory systems - no additives

    C. Circulatory systems - with additives

        1. Saline dilution
        2. Polyvinylpyrrolidone
        3. Pectin (Sure-Jell)
        4. Dimethyl sulfoxide
        5. Dextran (6% in saline)
        6. Gelatin

II. Photodynamic treatment of dogs

    A. Toxin

    B. Toxin-retention studies in dogs

    C. Phototreatment of poisoned dogs versus nontreated controls

There are two major divisions of our experimental work, in vitro systems and in vivo application to gs. The objectives were: (1) to determine whether photodynamic acti. could abolish the toxicity of botulinum toxin in the presence of mammalian blood, (2) to determine the effects of the type of irradition cell, the nature of the dye, and other experimental variables on the degree of destruction of the toxin by use of model systems simulating extracorporeal circulation of the blood, and (3) application of in vitro findings to treatment of the living dog that was poisoned by injection of toxin, using the technique of irradiating extracorporeally circulated blood.

Using these model systems, a number of variables have been tested that would have required a great deal more effort with the living animal. Several of the things learned from the model systems have been applied to photodynamic treatment of the living dog that was poisoned with the toxin; however, there are still several pieces of information that have not yet been applied to the treatment of the dog.

Our earliest experiments were performed with a static model system; that is, one in which the blood was not circulated but simply was confined in a small vial and subjected to irradiation (figure 49). The water bath and accessories are self-explanatory. Two floodlamps were used, one on each side of the bath. As a result, the light had to travel through about 6 in. of water before striking the sides of the vials. The rack containing the vials was agitated in a back-and-forth, longitudinal manner with a long metal rod attached to a Dubnoff metabolic shaking machine. Four mixtures, each lacking a single different component, were irradiated at one time, usually for 1 hr. The composition of a typical set of irradiated mixtures is shown in table 25.

The total volume of each mixture was 6 ml. Note that the rabbit blood formed 97% by volume of each mixture (except C). The concentration of MB, 0.1 mg/ml, was not arbitary but was chosen on the basis of several preliminary experiments. It does not necessarily represent the optimum concentration of dye. The concentration of toxin, 50 MU/ml, was such that subsequent injection of 0.2 ml of the mixture into mice introduced a calculated 10 MU per animal, a dose that is known to kill 100% of a group of mice during a 24-hr period. This procedure was adopted in preference to quantal assays, which are designed to determine the exact concentration of toxin remaining in the treated solution (i.e., LD50 determination). This approach resulted in a great saving in the time required to evaluate the effectiveness of a particular treatment.
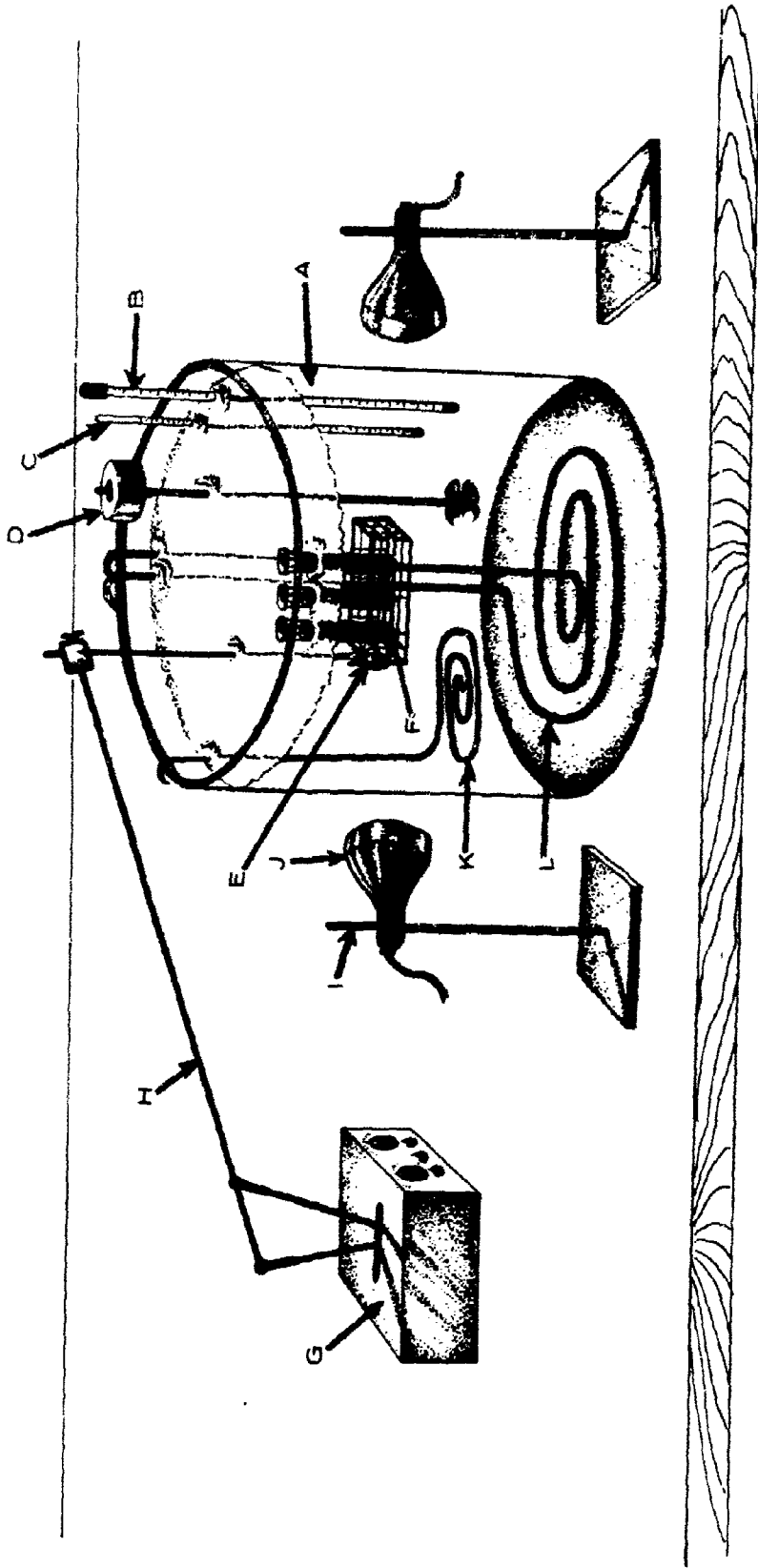
FIGURE 49

DIAGRAM OF STATIC MODEL FOR STUDY
OF PHOTODYNAMIC ACTION

A. Constant-temperature water bath
B. Thermoregulator
C. Thermometer
D. Electric stirrer
E. Rack for vials
F. Vials containing blood mixture

G. Shaking apparatus
H. Rod connecting shaker with rack
I. Lamp holder
J. GE floodlamps, 500 w
K. Heating coil
L. Cooling coil

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TABLE 25

COMPOSITION OF TYPICAL MIXTURES SUBJECTED TO PHOTODYNAMIC ACTION OF MB

Component	Stock concn	Vol of stock soln used in mixture				Final concn
		A	B	C	D	
		ml				
Rabbit whole blood	100% v/v	5.8	5.8	0	5.8	97% v/v
Saline	100% v/v	0	0.1	5.8	0.1	2% or 97% v/v
MB (in saline)	6 mg/ml	0.1	0	0.1	0.1	0.1 mg/ml
Botulinum toxin (in buffer)	3,000 MU/ml	0.1	0.1	0.1	0	50 MU/ml

As a control for the irradiated mixtures, an identical set of mixtures was kept in the dark for the same time under the same conditions of temperature and mechanical agitation. Table 26 shows the mortality in mice given 0.2 ml of each of these mixtures ip and observed for 2 days. Several interesting conclusions can be drawn from these results:

(1)   The photodynamic activity of MB abolished the toxicity of botulinum toxin in the presence of as much as 97% by volume of rabbit blood.

(2)   The photodynamic irradiation products of mixtures of rabbit blood, toxin, and MB were not toxic to mice when given ip.

(3)   The toxicity of botulinum toxin was not destroyed by MB in the dark.

The blood at the end of this period of irradiation was very dark purple and had a much greater viscosity. Some hemolysis probably had occurred.

TABLE 26

MORTALITY OF MICE INJECTED WITH TOXIN MIXTURES SUBJECTED TO PHOTODYNAMIC ACTION OF MB

Mixture	Omitted component	Mortality	
		Irradiated	Nonirradiated
A	Saline	0/10	9/10
B	Dye	10/10	9/10
C	Rabbit blood	0/10	9/10
D	Toxin	0/10	0/10

Similar results were obtained with mixtures containing 67% by volume of rabbit blood. Concentrations of 1 mg/ml of eosin, another well-known photodynamic dye, did not wholly destroy the toxin in the presence of rabbit blood, but did so in aqueous solution.

No particular studies on the relationship between time of irradiation and degree of destruction of toxin were performed with this apparatus. The period of irradiation (1 hr) was not wholly arbitrary; it was to some extent based on the results of Weil and coworkers,[2] who showed that the toxin was 99.99% destroyed at the end of 42 min in buffered aqueous solutions containing 0.03 mg/ml of MB. The aim at this time was a qualitative one; i.e., to see if destruction of the toxin could be accomplished in the presence of mammalian blood by photodynamic action.

To more realistically simulate the application of the photodynamic technique to treatment of the living animal, in vitro models were used, in which a blood mixture containing toxin and dye was circulated rapidly under a light field. A sketch of the experimental setup is shown in figure 50.

In this apparatus, a mixture of dog blood and known concentrations of dye and toxin was circulated from a reservoir through an irradiated cell in a water bath and back to the reservoir by means of a Sigma-motor pump.
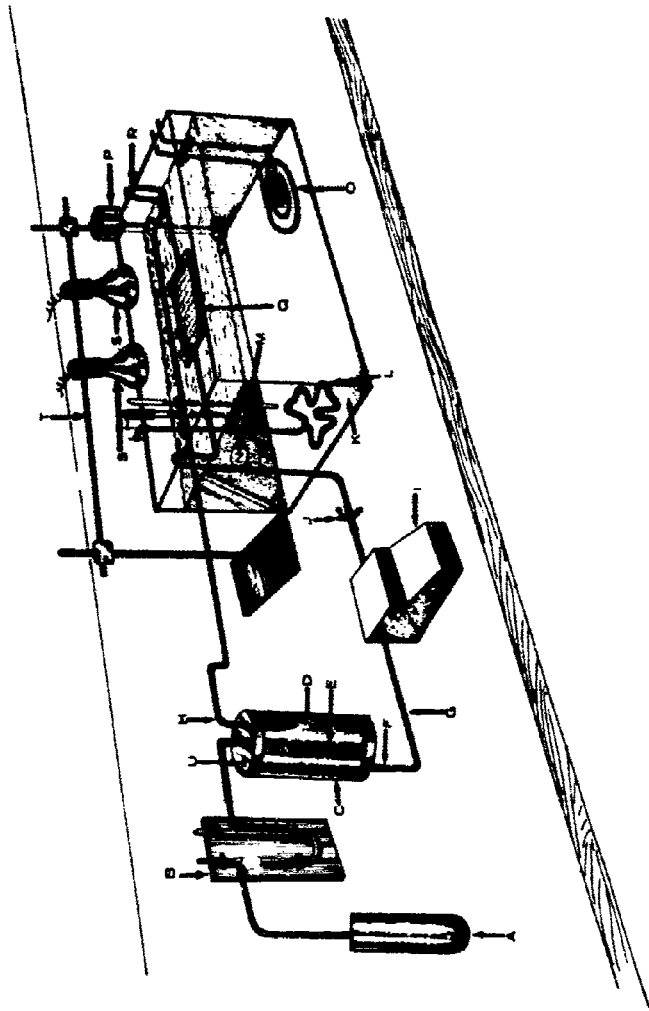
FIGURE 50

DIAGRAM OF CIRCULATORY APPARATUS FOR STUDY
OF PHOTODYNAMIC ACTION

A. Oxygen tank
B. Orifice flowmeter
C. Blood-mixture reservoir
D. Siliconized stainless-
   steel packing
E. Oxygen gas dispersing
   tube
F. Draining stopcock
G. Connecting tube between
   reservoir drain and pump

H. Blood-mixture return tube
I. Circulating pump
J. Two-way sampling valve
K. Constant-temperature
   water bath
L. Heating coil
M. Thermometer
N. Thermoregulator
O. Cooling coil

P. Electric stirrer
Q. Irradiation cell
R. Connecting tubing between
   cell outlet and blood-mixture
   return tube
S. Photoflood lamps, 500 w
T. Lamp holder
U. Oxygen vent

Oxygen was slowly bubbled through the blood mixture in the reservoir during the experiment. Samples of the irradiated blood mixture were drawn off at definite time intervals through the sampling valve (J). The toxicity of each sample was assayed by ip injection into groups of 10 mice by the nonquantal screening procedure used in the static-model experiments mentioned earlier.

Table 27 shows the results obtained with MB and with hematoporphyrin hydrochloride (HPN). The gradual decrease in toxicity with length of sampling time, showing the progressive inactivation of the toxin, can be observed. The differences in the minimum time required for zero mortality are due to differences in the volume of the irradiation cell and also to whether or not the blood was diluted with saline. These points will be elaborated later.

TABLE 27

INACTIVATION BY PHOTODYNAMIC DYE OF TOXIN IN DOG BLOOD

Dye	Mortality in mice							
	Before irradiation	7.5 min	15 min	30 min	45 min	60 min	90 min	120 min
MB a, b/	9/10	10/10	9/10	8/10	—	10/10	—	1/10
MB	10/10	—	—	10/10	—	0/10	0/10	0/10
MB	8/10	—	10/10	9/10	10/10	9/10	0/10	0/10
MB	10/10	—	10/10	9/10	0/10	0/10	0/10	0/10
HPN c/	9/10	—	5/10	0/10	0/10	0/10	—	—

a/ MB, 0.1 mg/ml.

b/ 0/10 at 240 min.

c/ HPN, 1.0 mg/ml.

HPN appears to be much more effective photodynamically than MB, but this may be due simply to the higher concentration of HPN, 10 times that of MB.

Several types of irradiation cells (figure 50, Q) were designed and were tested in this apparatus. These included glass coils of various diameters, glass capillary tubes, and glass and plastic (Lucite) cells of shallow depth. For most of the in vitro studies, a flat band-type glass cell, 3.4 mm deep, 170 mm long, and 29 mm wide, was used. The internal volume was 16 ml. It is apparent from spectrophotometric theory that the thinner the layer of blood exposed to the light, the less the absorption of light energy by the blood pigments, and the greater the probability of activating the number of dye molecules required for destroying the toxin. One of our major efforts is the design of cells and other apparatus that will yield microthin layers of blood for irradiation. The intensity of the incident light at the desired distance from the cells has been measured with a Weston light meter and was found to be greater than 12,000 foot-candles, the capacity of the meter. We intend to attenuate the light with Inconel filters, so that the light intensity will be measurable on the Weston meter.

To determine the length of time the whole blood sample was irradiated, we used the formula given below. This formula can be derived from basic considerations. It shows that the time of irradiation of the total volume of blood depends only on the ratio of the volume of the irradiation cell to the volume of the blood sample and the experimental period, and is independent of the circulation rate. The time of contact of the sample with the irradiation cell during one cycle, however, is obviously dependent upon the circulation rate and the volume of the cell.

$$T_i = \frac{C \times E}{V}$$

where

T_i = total irradiation time of total blood sample

C = volume of irradiation cell

E = time of experimental period (total circulation time)

V = volume of blood sample

There are many other variables in a study of this kind: light intensity, rate of flow of oxygen, rate of flow of blood mixture, dye concentration, etc. The composition of typical mixtures that were tested is shown in table 28.

TABLE 28

COMPOSITION OF TYPICAL MIXTURES SUBJECTED
TO PHOTODYNAMIC ACTION OF MB

Component	Stock concn	Concn of components in mixture		
		A	B	C
Dog whole blood	100% v/v	93% or 75% v/v	93% v/v	75% v/v
Saline	100% v/v	7% or 25% v/v	7% v/v	25% v/v
MB (in saline)	5 mg/ml	0.1 mg/ml	0.1 mg/ml	0.1 mg/ml
Botulinum toxin (in buffer)	50,000 MU/ml	200 MU/ml	200 MU/ml	200 MU/ml
Additive (in saline)	Varies	None	Present	Present

The total volume of the blood mixture was usually 100 ml. The toxin concentration was increased from 50 MU/ml in the static system to 200 MU/ml in this one. A very small amount of buffer solution was always added to the mixture, since it was a solvent for the dye as well as the additives. In the B series of mixtures, the total saline was 7% by volume. It was increased to 25% in the C series. The A series of mixtures contained either 7% or 25% by volume of saline but no other additive and, thus, served as controls for the other two series. In some early experiments without additives, the concentration of saline was less than 7%.

You will note the reference to additives in table 28. The justification for adding something to the blood in addition to dye and toxin is found in a report by Bellin,[8] who points out that the adsorption of dye molecules onto surfaces of high molecular weight profoundly affects their photochemical properties. Among numerous examples she describes is that of the dye Rose Bengal, which shows strongly enhanced photosensitizing properties, as measured by the hemolysis of red cells, when bound to polyvinylpyrrolidone (PVP). The potentiating effect of several high-molecular-weight substances

on the photodynamic efficiency of MB was, therefore, studied. We have done more work, to date, with PVP than with any other polymer. Table 29 compares test results using PVP of various molecular weights with those where no additive was used.

The results in table 29 show a slight potentiation of the photodynamic efficiency of MB by PVP, in terms of the calculated irradiation time required to effect zero mortality in the mice. Sharper sampling intervals might have shown greater differences in the required irradiation time between controls and PVP-containing mixtures. The molecular weight appeared to have no effect, and doubling the concentration of PVP did not have much effect, either.

Table 30 shows the results with other high-molecular-weight additives. The results of experiments 2, 6, 7, and 10 from table 29 are repeated in this table for comparison.

The minimum irradiation time for zero mortality was 6.8 min when pectin was added and the blood was diluted 3:1 (experiment 8). Dilution plus 6% dextran was almost as effective. These additives have not as yet been tried in vivo with the dog. All of these additives, with the exception of dimethyl sulfoxide (DMSO), have been used as human-plasma volume expanders in emergency treatment of shock, burns, etc. They should, therefore, be acceptable as priming fluids for extracorporeal admixture with blood.

We also did experiments on photodynamic treatment of dogs poisoned with botulinum toxin. The experimental setup is shown in figure 51. The right side of this setup is similar, if not identical, to the corresponding one in the previous in vivo diagram. In addition to the presence of the dog, the major difference is the dye-infusion pump used for continuous infusion of MB into the extracorporeal circuit. The necessity for this continous infusion arises from the fact that MB is rapidly reduced to the leuco- base in vivo (probably by the liver) and has to be constantly replenished in order to maintain a steady photodynamic action, although it does function as a true photochemical catalyst, the mass of which is unchanged by photodynamic reaction. For in vitro model systems, MB was simply premixed with the blood and was circulated for the duration of the experiment. For photo-sensitive dye having no redox properties, a single iv injection should suffice for in vivo experiments.

TABLE 29

POTENTIATION OF PHOTODYNAMIC EFFICIENCY OF MB BY PVP

Expt No.	Mol wt of PVP	Concn of PVP	Concn of saline	Vol of cell	Earliest sample time for zero mortality a/	Irradiation time for zero mortality b/
		gm/100 ml	ml/100 ml	ml	min	
1	—	None c/	2	16	240 d/	38.4 e/
2	—	None	7.4	16	60	9.6
3	40,000 f/	0.9	7.4	16	30 g/	8.7
4	40,000 f/	2.3	7.4	16	60	9.6
5	40,000 f/	1 h/	7.4	16	60	9.6
6	—	None	7.4	16	90	14.4
7	40,000 f/	1	7.4	16	60	9.6
8	360,000	0.9	24.6	22.5	45	10.1
9	10,000	0.9	24.6	22.5	50	11.3
10	—	None	24.6	22.5	45	10.1

a/ Observation period of at least 4 days.

b/ Calculated by formula $T_i = \dfrac{C \times E}{V}$.

c/ Blood mixture contained 10.2 mg of zinc oxide/100 ml.

d/ Mortality was 10% at 120 min.

e/ One 500-w lamp and one 150-w lamp were used.

f/ Molecular weight was not given; presumed to be 40,000.

g/ Volume of blood mixture was 55 ml.

h/ Contained 0.1 mg of MB·$ZnCl_2$/ml instead of MB.

TABLE 30

POTENTIATION OF PHOTODYNAMIC EFFICIENCY OF MB BY HIGH-MOLECULAR-WEIGHT ADDITIVES

Expt No.	Additive	Concn of saline	Concn of additive	Vol of cell	Earliest sample time for zero mortality	Irradiation time for zero mortality
		ml/100 ml	gm/100 ml	ml	min	
1	None	7.4	—	16	60	9.6
2	None	7.4	—	16	90	14.4
3	None	24.6	—	22.5	45	10.1
4	PVP	7.4	1	16	60	9.6
5	DMSO	None a/	10 b/	16	90	14.4
6	Pectin c/	7.4	0.2	16	60 d/	9.6
7	Pectin c/	7.4	0.6	16	60 d/	9.6
8	Pectin c/	24.6	0.9	22.5	30	6.8
9	Dextran	24.6	1.4	16	45	7.2
10	Gelatin	24.6	0.2	22.5	45	10.1

a/ No additional solvent was needed, since DMSO is liquid.

b/ DMSO, 10 ml/100 ml of mixture.

c/ "Sure-Jell," a mixture of pectin, dextrose, and fumaric acid.

d/ Mortality was 10% at 45 min (4 days).
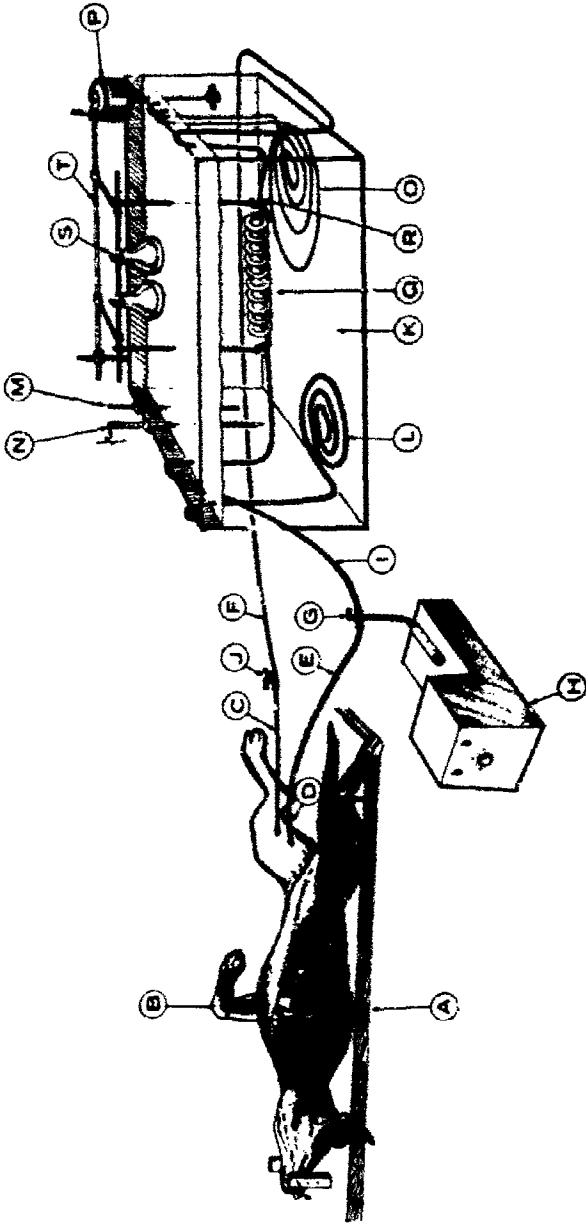
FIGURE 51

DIAGRAM OF EXPERIMENTAL APPARATUS FOR PHOTODYNAMIC
TREATMENT OF DOG BLOOD

A. Operating table
B. Dog
C. Cannula from femoral vein
D. Cannula from femoral artery
E. Tygon tubing connecting arterial cannula to dye-infusion adapter
F. Tygon tubing connecting venous cannula to sampling valve

G. Dye-infusion adapter
H. Dye-infusion pump
I. Tygon tubing connecting dye-infusion adapter to irradiation cell
J. Three-way sampling and injection valve
K. Constant-temperature water bath
L. Heating coil
M. Thermometer

N. Thermoregulator
O. Cooling coil
P. Electric Stirrer
Q. Irradiation cell
R. Tygon tubing connecting cell to sampling valve
S. Photoflood lamps, 500 w
T. Lamp holder

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The experimental approach was, for the most part, the same in all experiments and comprised the following steps:

1. Anesthetization (sodium pentobarbital).

2. Heparinization.

3. Priming of irradiation cell and rest of extracorporeal circuit with saline or 5% dextrose-saline.

4. Cannulation of femoral artery and femoral vein.

5. Formation of closed extracorporeal circuit.

6. Determination of rate of flow of blood in extracorporeal circuit.

7. Injection of toxin into blood flowing in extracorporeal circuit.

8. Immediately after injection of toxin, slow infusion of a saline solution of MB by means of a peristaltic-action pump.

9. Passage of the blood-dye-toxin mixture through a glass or plastic irradiation cell.

10. Irradiation of the blood in the cell by visible light using one to three 500-w photoflood lamps.

11. Sampling of the blood at selected time intervals.

12. Bioassay of residual toxin in the blood.

13. Determination of MB concentration in the blood.

14. At the end of the experiment, im injection of the dog with procaine penicillin and irrigation of the incised area with the solution before suturing.

15. Observation of the treated dog and comparison of its survival time with that of an untreated dog (control).

In the earlier experiments, a calibrated, dual-float rotameter was used in the extracorporeal circuit to measure the rate of flow of the bloodstream (step 6). This was later discarded and replaced by the simple procedure of timing the flow of a small volume of blood into a graduated cylinder.

The concentration of the MB solution and its rate of infusion were adjusted so that, on the basis of the rate of flow of blood in the extracorporeal circuit, a concentration of 0.1 mg of dye/ml of blood would be approximated. The concentration of dye in the blood was measured by an acetone-extraction method developed in our laboratories.

Although priming of the extracorporeal circuit has been performed with saline or dextrose-saline, any of the plasma-volume expanders that have been tested in vitro might be used for this purpose—once it is shown to be physiologically compatible with the living dog.

The toxin was injected into the blood by means of a three-way valve inserted in that part of the circuit leading from the irradiation cell to the femoral vein. Samples of blood for bioassay of residual toxin and for measurement of MB concentration were removed from the circuit through this valve.

The volume and design of the irradiation cells were varied in these experiments in order to determine the most efficient type for photodynamic action. These cells included glass coils and tubes and a specially designed cell of shallow depth constructed of Lucite. Other designs are on the drawing board or are being contemplated.

In later experiments, the height of the photoflood lamps was adjusted so that 7,000 foot-candles of light energy, as measured by a Weston light meter, impinged on the surface of the water bath. The cell was submerged under 1 in. of water. In order to evaluate the effectiveness of the photodynamic treatment (aside from survival of the animal), samples of blood were taken at intervals from the animal while it was under treatment and were bioassayed for residual toxin. Bioassays were conducted by the method of quantal response (i.e., the LD50 of a series of progressively diluted blood samples was determined by the method of Bliss), and the concentration of toxin in the sample was measured. As a control study, equivalent doses of toxin were injected into dogs that received no further treatment. Whole-blood samples were taken from these dogs at intervals of time corresponding as closely as possible to those taken from dogs undergoing photodynamic treatment and were bioassayed for residual toxin. In one instance, the blood sample was centrifuged to yield plasma, and the plasma was bioassayed.

Table 31 compares the residual toxin found in two control dogs and one treated animal. The footnotes indicate some of the difficulties encountered in these assays. At similar time intervals, the residual toxin in the treated dog is less than that in either of the controls. This shows that the rate of detoxification was enhanced by the photodynamic treatment, but not to the extent desired. Since the injected dose was 5.6 LD50's, 20% of the original concentration remaining after 90 min still represents 1 LD50. This could account for some clinical signs of botulism that we saw in the treated dogs.

The results for the two control dogs agree for similar time intervals. Since whole-blood samples were bioassayed for dog No. 7 and plasma samples for dog No. 13, the suggestion is that the iv-injected toxin is not concentrated in the red cells but rather in the plasma. Supplementary experiments (known amounts of toxin were added to whole blood that was subsequently centrifuged to yield plasma, which was then bioassayed) showed that the added toxin was almost fully recoverable from the plasma.

Assumptions were made in table 31 with respect to the magnitude of the blood and plasma volumes in the dog. Henceforth, the actual volumes will be measured by the classical Evans blue procedure. Some values recently obtained for blood volume lie in the range of 90 to 100 ml/kg, instead of 70 ml/kg.

To date, five dogs have been given photodynamic treatment with MB after iv injection of toxin. Three received 15,000, one 12,000, and one 9,000 MU/kg of toxin. Five corresponding control dogs were given equivalent doses of toxin, but received no other treatment.* Another dog was subjected to the entire treatment, but with the omission of toxin.

Table 32 shows that the survival time of three dogs undergoing photodynamic treatment after poisoning with 5.6 LD50's of botulinum toxin ranged from 4 to 12 days, compared with a survival time of 2 days for three untreated controls. Dog No. 2, the control for treated dog No. 3, received 9,000 MU/kg, equivalent to 3.3 LD50's, and survived 11 days, after which it was killed. This dog did not appear to be moribund at any time, although it did show some mild toxic signs of botulism. The treated dog No. 3, which received the same dose of toxin plus photodynamic treatment, was killed 10 days later. It showed no toxic signs of botulism during this period of observation.

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* These controls are not the same dogs mentioned earlier, from which blood samples were taken and assayed for residual toxin.

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TABLE 31

DISAPPEARANCE OF TOXIN FROM BLOOD OF
PHOTODYNAMICALLY TREATED
AND CONTROL DOGS

Sampling time interval	Residual toxin, percentage of zero-time concentration		
	Control No. 7 a/	Control No. 13	Control No. 9
min			
3 - 8 b/	—	68 c/	—
15 - 18	67 d/	67	49 d/
30 - 34	—	52	35
60 - 65	49	60	21
90 - 91	—	44	20
119 - 140	48	46	—
240 - 245	36	38	—

a/ Concentration at zero time always equaléd 214 MU/ml of whole blood for constant dose of 15,000 MU/kg, and assumption of blood volume was 70 mg/kg.

b/ Grouped time intervals were used rather than actual time sample being taken, since sample times for controls did not always match those of treated dogs.

c/ Blood samples from this dog were centrifuged to yield plasma and were bioassayed. Concentration at zero time was 357 MU/ml of plasma when assuming plasma volume was 42 ml/kg.

d/ Statistical analysis could not be made because of only one partial mortality fraction. Value shown is mean of estimates from mortality fractions above and below this partial. Whole-blood samples were bioassayed.

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TABLE 32

FATE OF DOGS UNDERGOING PHOTODYNAMIC TREATMENT AFTER IV POISONING BY BOTULINUM TOXIN

Dog No.	Exptl group	Irradiation cell			Dose of toxin	Treatment			Fate
		Type	Depth	Vol	MU/kg	MB	Total time	Irradiation time	
			mm	ml		mg	min	min	
1	Control	Coil	12	79	None	840	58	3.9	Survived; good clinical condition
2	Control No. 3	–	–	–	9,000	–	–	–	Survived; mild toxic signs
3	Treated	Coil	8	29	9,000	450	65	1.7	Survived; no toxic signs
6	Control No. 5	–	–	–	15,000	–	–	–	Died 2 days later; toxic signs
5	Treated	Coil	12	143	15,000	64	72	10.1	Died 12 days later; delayed toxic signs
8	Control No. 9	–	–	–	15,000	–	–	–	Died 2 days later; toxic signs
9	Treated	Tubes	12	ca 100	15,000	918	90	10.7	Died suddenly 4 days later; not due to toxin
12	Control No. 11	–	–	–	15,000	–	–	–	Died 2 days later; toxic signs
11	Treated	Tubes	18	ca 200	15,000	378	60	10.7	Died 4 days later; toxic signs
15	Control No. 16	–	–	–	12,000	–	–	–	Died 2 days later; toxic signs
16	Treated	Plastic	2.5	ca 220	12,000	ca 1,340	70	13.2	Died 1 day later

Although the treated dogs survived longer than the untreated ones, some toxic signs of botulism were observed in all except dog No. 3. Furthermore, it appeared that the cause of death of treated dogs No. 9 and No. 16 was not the toxin. Dog No. 16 also received a transfusion of 240 ml of dog blood after the photodynamic treatment. It is not know if incompatibility of the donor's blood may have contributed to the early death of this dog.

An autopsy was performed on dog No. 9. The final diagnosis showed so many pathological conditions that the overt cause of death could not be attributed to a specific one.

We are aware of the destructive effects on the blood elements of photosensitive dyes, such as MB. The work of Smetana[9] at Columbia some years ago with hematoporphyrin showed that it is the plasma proteins that are principally affected by photodynamic action, whereas the erythrocytes are resistant. In photodynamic treatment of whole blood, therefore, the plasma proteins would tend to protect the red cells from the destructive effects. What about the products of photodynamic oxidation of the plasma proteins, however? Are they toxic? Hundreds of mice have been injected with irradiated mixtures of dye, toxin, and dog blood, and the only toxic signs observed were those of botulism. In surviving groups of mice given mixtures that had received sufficient irradiation to destroy all the toxin, no signs were observed. Furthermore, 100 ml of blood was withdrawn from a dog, mixed with MB and saline, circulated and irradiated in the photodynamic apparatus of figure 50 for 94 min, and then reinjected into the same dog. No toxic signs were observed. Dog No. 1 (table 32), which was subjected to the complete photodynamic treatment but with omission of the toxin, survived with no ill effects. All of these observations suggest that the products of photodynamic action of the dye on the blood are not toxic to the host.

We believe that the failure to achieve 100% survival of the dogs is due to the design of the irradiation cells, which have not been large enough and thin enough. The band-type cell, which has been so effective in the in vitro model experiments, has not as yet been tried for photodynamic treatment of the dog. This is because of the difficulty in fabricating a large and thin glass cell. We have, however, recently completed a design for a cell that has been submitted for construction by commercial fabricators.

For the purposes of increasing the efficiency of the photodynamic action, decreasing the harmful effects on the animal, and achieving rapid and

complete destruction of toxin in the blood, the following efforts are being pursued:

(1)   Irradiate the blood in as thin a layer as possible to facilitate penetration of the light energy.

(2)   Enlarge the irradiation cell to as large a volume as possible that is compatible with the blood volume of the animal.

(3)   Decrease the contact time of the flowing blood with light and dye in the irradiated cell to as short a time as possible that is consistent with sufficient time for inactivation of the toxin.

(4)   Increase the intensity of the radiant energy to as high a level as possible that is consistent with keeping damage to the blood elements to a minimum.

We are fully cognizant of the fact that, in treating poisoned dogs photodynamically, the experimental conditions have been optimized by infusing the dye immediately after iv injection of the toxin. Such treatment may not be a realistic one, in view of the usual slow absorption of toxin into the bloodstream from the GI tract. This form of poisoning, however, will be subjected to photodynamic treatment following manifestation of the toxic syndrome in the dog.

Whether or not we succeed in achieving 100% survival of poisoned dogs, we believe that the photodynamic mode of treatment is a fresh approach to therapy of other toxemias.

DISCUSSION

Dr. Zacks (The Pennsylvania Hospital): This sounds like a very useful approach, but I wonder if you are aware of one or two facts related to the use of MB. First of all, MB is as effective an antiChE as physostigmine in very low concentration.

Mr. Feinsilver: Not in vivo, in vitro.

Dr. Zacks: In the proper dose, cholinergic signs occur in animals injected with MB. Your control experiment, however, would tend to make one wonder whether this effect occurred under the conditions of your experiment. If this treatment is used in therapy of poisoning, I think one must be aware that the symptomatology that could follow ChE inhibition due to MB would confuse your interpretation of whether the muscle weakness was due to botulinum toxin or to ChE inhibition with block. The characteristic response of isolated muscle to MB in vitro is an initial potentiation at low concentrations followed by a very clear, but reversible, block. This can be demonstrated in vitro quite easily and certainly does occur in mice, rats, and dogs in vivo. Also intravascular injection of Janus green B, which is a dye quite similar to MB in having a resonance-produced quaternary ammonium group, produces salivation, bradycardia, and vomiting in dogs. I would suggest that it might be useful to try other dyes, such as eosin, which do not have these properties. I would call your attention to the papers of Renz[*] and Massart and Dufait, [**] where these dyes are listed with their properties. A table is also included in The Motor Endplate.

Mr. Feinsilver: I am aware of the various photodynamic efficiencies of these different dyes. As I pointed out in the paper, eosin is not effective in detoxifying blood. You can't get it to destroy the toxin in blood. MB is by far one of the most effective photodynamic dyes known. I don't say it is the most effective; it is one of the best.

Dr. Zacks: Have you any experience with dyes of the crystal violet group, which have a triazine structure rather than the two rings, as in MB? This has much less ChE-inhibiting activity than MB.

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[*] Renz, E. Methlenblau und cholinesterase. Arch. Exptl. Pathol. Pharmakol. 196, 148 (1940).

[**] Massart, L., and Dufait, R. P. Hemmung der Acetylcholin-Esterase durch Farbstoffe und durch Eserin. Enzymologia 9, 364 (1941).

Dr. Hassett (CRDL): Aside from that factor, there is also the possibility that there might be differences because of the absorption of light by various dyes. Have you taken this into account?

Mr. Feinsilver: In what respect?

Dr. Hassett: Each dye has a typical absorption curve, and a given dye may be more or less effective in absorbing light.

Mr. Feinsilver: Right. Of course it is the action spectrum, the absorption curve when it is mixed with something else. We mixed the dye with blood. The absorption spectrum of the mixture is a function of the resultant color. You are encountering absorption at a wavelength of a mixture of so much MB and so much hemoglobin. Apparently the MB is still effective; i.e., its action spectrum is still effective when mixed with blood, otherwise we wouldn't have gotten the results we did. Normally, the peak efficiency of a dye is at the peak of its absorption spectrum in aqueous solution, which is about 670 mμ for MB. It may be slightly modified when mixed with another material that is also highly absorbent, but it was not seriously affected in this case.

Dr. Sheff (The Pennsylvania Hospital): Did you test toxicity?

Mr. Feinsilver: No.

Dr. Zacks: At the recent Federation Meeting, Boroff and coworkers* showed a decrease in toxicity after photooxidation of the toxin.

Mr. Feinsilver: Weil has done some work on that. He has photooxidized botulinum toxin and MB in a buffered aqueous medium and shown that its precipitin action is retained up to a certain point. Its antigenic properties are still retained if the oxidation is not carried too far. We have a reprint of Weil's work on that.

Dr. Tyler (Peter Bent Brigham Hospital): One of the assumptions in this type of treatment is that botulinum toxin in the blood is damaging, to the patient. There are a few observations in the literature of patients who have recovered in spite of large amounts of active botulinum toxin in the blood. I was wondering if you have any observations in dogs to confirm that free toxin in the blood may exist at the time of clinical recovery?

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* Boroff, D. A., Das Gupta, B. R., and Fleck, U. Relation of Trypotophan to Biological Activity of Botulinum Toxin. Federation Proc. 23, 2251 (1964).

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Mr. Feinsilver: I am sorry to say that we have not. There is much more work to be done, especially in hematology. We have only made observations on the dogs, with no clinical tests yet. We hope to obtain some survivals, which will be thoroughly studied.

Dr. Riesen (IITRI): The reactants in the system are the dye, the toxin, and oxygen, with light energy. You have now discussed three of these; perhaps I missed something on oxygen. Have you depended solely on the oxygen content of the air, or have you increased the oxygen content—used pure oxygen rather than air? If so, has this any effect on the system? If you do plan to use pure oxygen, I would like to point out the effect of this on certain structures in the animal due to hyperoxia. Myokinal systems, among others, are directly affected by oxygen. I would be interested in your comments on this factor.

Mr. Feinsilver: We have not controlled the oxygen or the atmosphere. There are many experiments (among which the work of Smetana has been outstanding) on the effect of oxygen level on photodynamic efficiency of hematoporphyrin, which may or may not be a good prototype for MB. A certain minimum is needed, and after a certain level is reached, excess oxygen is of no value in increasing the photodynamic efficiency. In the in vitro models, we bubbled it slowly through the blood reservoir. In dogs, we just relied on the normal oxygenation process. Of course, they could be put in pure oxygen, or given forced ventilation. In view of earlier work on its effect, I don't believe it is as important as it may seem.

Question: Inaudible.

Mr. Feinsilver: Yes, the MB is supposed to act as respiratory catalyst. It oxidizes homoglobin to methemoglobin and also can reduce methemoglobin back to hemoglobin. Its use is cyanide poisoning is well known.

Question: Inaudible.

Mr. Feinsilver: We have not gone into biochemical mechanisms to the extent we would like.

Dr. Petty (Maryland Medical-Legal Foundation, Inc.): One of the nicest ways of getting a thin film of blood is simply to collect it in a plastic bag. The blood flows between the two plastic layers. I wonder if you might not consider this for your cell. It is a good way to get a broad surface and a thin layer.

Mr. Feinsilver: Thanks for suggesting it. We are looking for such suggestions.

Dr. Sheff: If you are presuming that it is the singlet state of oxygen that is operative here, I wonder if you would get similar results by diluting the oxygen that you bubble through the system with very, very low concentrations of ozone, which, under these conditions, would give you an equivalent of a singlet state of the oxygen atom.

Mr. Feinsilver: From what I know of ozone, it is pretty rough on organisms.

Dr. Sheff: I specifically mentioned extremely low concentrations.

Mr. Feinsilver: I see, yes. I would tend to shy away from anything like that.

Mr. Vocci: There is no question that a study of photosensitive dyes in the system that we have discussed is quite in order. We are well aware of the antiChE properties of the phenothiazine dyes. We have not measured their equilibrium constants, but MB, I believe, would be classed as a pseudoChE inhibitor. Insofar as eosin is concerned, in the absence of blood, the pre-irradiation of eosin will give a dark reaction and disruption of the toxin. Eosin itself, however, is extremely destructive to the blood elements, either preirradiated or concomitantly irradiated. It tends to attenuate the albumin considerably. The work of Bolande, who evaluated the sister dye of MB, toluidine blue, and eosin, shows that there was a very small attenuation of the albumin fraction in plasma with toluidine blue. Eosin, however, had a massive destructive effect and considerably altered the albumin:globulin ratio, in addition to being cytotoxic. Both irradiated and preirradiated eosin produced a toxic syndrome, whereas we were looking for photodestruction of the toxin in blood. MB will do the same thing if the whole animal is irradiated after the dye is injected. We have observed the very typical hematoporphyrin-shocklike syndrome with MB in the animal. This is the reason for establishing an extracorporeal circulation, because this syndrome is abolished by treating the toxin in a compartment outside the animal. Insofar as these studies are concerned, we plan to investigate these dyes by measuring their oxygen uptake in a Warburg apparatus and determining from this to what degree the toxin is inactivated. This will not be easy and will require some manipulation of the Warburg apparatus concomitantly with a photodynamic setup.

Mr. Feinsilver: Extracorporeal circulation seems to be in much favor with some investigators at Brookhaven, especially Dr. Cronkhite, for treatment of leukemia patients. It is not exactly a photodynamic treatment, but they

adopted the idea of treating extracorporeally circulated blood with X-rays. They find that this extracorporeal treatment is much less harmful to the patient. The side effects are much less. It is also found that destruction of leucocytes still occurs when the patient becomes drug-resistant.

Dr. Tyler: There is a fair amount of literature, dealing with transplantation in human beings, which has many problems similar to these. The same problem of finding a good dialysis setup is fairly well worked out for hemodialysis in human beings, where you can choose the pore size of the filter and where there is a bath with circulation of the blood outside the body through various lengths or thicknesses of tubing. Many are commercially available in small packages. In fact, patients now do these procedures at home with simple plug-in units. I think these units could be easily adapted to this procedure. Attempts have been made to eliminate the antigen-antibody reactions in these patients. They have removed and stored bone marrow. After X-rays have destroyed all the blood elements, transfusion of the stored bone marrow has been attempted with partial success. Some of these techniques may well be applicable to these experiments. If the patient was once exposed to botulin, we really don't know if it would make any difference if you took all the botulin out of the blood. I would expect that it would do some good, but this is pure presumption.

Mr. Feinsilver: Thank you.

Dr. Serrone (Albany Medical College): Years ago, we studied the effect of photosensitivity on such substances as atabrine and 4-aminoquinolines in general, and the 8-aminoquinolines in malaria. We found that it is not just a question of light. The photofloods that you used radiate visible light, IR, and, perhaps, UV, so that it is important for you to ascertain which parts of the spectrum are actually responsible for the phenomenon you are studying. Much heat was generated by these lights, and this is something that has to be considered, so we used Kelvin units instead of foot-candles. Kelvin units take into account the brightness of the light with the amount of heat produced. I didn't notice the temperature of the water bath and how you knew if any of the IR was heating the coil. Is there any such effect in this system?

Mr. Feinsilver: The water bath was held at 37.5°C. There was a heating coil and a cooling coil. I agree that the heat from the lamps is terrific. We had trouble from almost cooking the blood in the large plastic cell, because the plastic is more transparent to UV light than glass is. Ordinary glass stops much of the UV energy. When we used Lucite, in a 2.5-mm sandwich, we did cook the blood somewhat and blocked the whole thing. The

whole experiment was stopped. On that basis, I decided not to use plastic again. The effective part of the light is the so-called action spectrum, which I have not actually checked with MB in blood, but it is not far from the action spectrum in aqueous solution, about 670 mμ. This is the effective portion according to photodynamic theory. There isn't much point in trying to use monochromatic light, as the most effective wavelengths of MB when mixed with blood would have to be found. Just use the white light.

Dr. King: It seems to me that it would be simple to get the approximate maximum of both the MB and the combination of MB and blood. It is true that the combination of these maxima would probably be the most efficient, as far as your process is concerned. Then, if you used monochromatic light, you would not be concerned with the cooking effect of the IR.

Mr. Feinsilver: Thank you.

Mr. Vocci: We have often measured the spectra of MB in blood plasma. Mr. Feinsilver was just not aware of this. As a matter of fact, we found that MB complexes with bilirubin in blood, and, with this complex, there is a shift in the spectrum. It shifts from 670 mμ down to about 650 mμ. It is a small shift, but it can be picked up very readily in a DK-2. This complex with bilirubin and the shifting have been reported in the literature. Presumably the action spectrum is in the 650- to 670-mμ region.

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PHARMACODYNAMICS OF BOTULINUM POISONING

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The title is somewhat pretentious, because I am going to try to confine my remarks to the cardiovascular system. The term "general pharmacodynamics" involves a great deal more than I am prepared to discuss. I would like to begin with a small apologia or explanation of why anyone would be concerned with the study of cardiovascular pharmacodynamics of the toxin, since all of you have already been exposed to the fact that its locus of action, or the locus of action that has been most discussed, is at the neuromuscular junction. Under ordinary circumstances, if the toxin is used in any concentration approaching the lethal dose for an animal, that animal will die of paralysis of the respiratory apparatus before any cardiovascular effects can be observed. Therefore, under typical experimental circumstances, the cardiovascular effects, if there are any, are masked by the intervention of the effects of the toxin at the neuromuscular apparatus.

There is much suggestive evidence in the literature that cardiovascular effects of the toxin do exist and that they may, in terms of the survival of the individual, be extremely important. In some experiments performed in the 1950's, particularly by Guyton, animals under artificial respiration (closed chest), when given appropriate doses of the toxin, died of what he attributed to a cardiovascular syndrome. In this situation, of course, paralysis of the respiratory apparatus has no effect on survival. He observed the development of heart blocks and dysrhythmia and detected what appeared to be a decrease in peripheral resistance. It occurred to him that there was, indeed, a cardiovascular pharmacology of the toxin, but he did very little beyond these observations. He did make one very interesting observation, which was that he could seemingly prevent the death of the animal if he infused a sympathomimetic amine, paradrine, with the toxin. For those not familiar with paradrine, it is a pressor amine of very low potency. But, as Guyton used it, he was able to maintain the mean arterial blood pressure during the course of action of the toxin, and he suggested that a possible mechanism of death, attributable to the toxin in a situation where respiration was available, could be overcome by maintaining a normal peripheral circulation.

In addition, there has been a series of publications in the Russian literature that describes experiments with the isolated, perfused heart, showing that botulinum toxin, in relatively high concentrations, is capable of

producing inotropic effects in this type of preparation, which they ascribe to a direct effect on the contractile elements of the heart muscle. Also, in my own scanning of case histories of intoxication in man, where artificial respiration and other supportive measures were employed, the individuals proceeded to what appeared to be cardiovascular death. If this is true, it seems profitable to investigate the cardiovascular pharmacodynamics of the toxin. This is really the rationale for performing the few experiments I am going to discuss.

First, I will describe experiments that were done on the isolated, perfused, heart of the cat. I will describe them schematically. The cats were anesthetized with sodium pentobarbital, the chest was opened, and the heart was excised and put into a beaker of physiological salt solution where it was allowed to beat so that the coronary supply cleared itself of blood. Clotting at a later time was thereby avoided. The setup used required perfusion of the coronary vessels by tying a cannula into the aorta so that pressure was applied in the aorta; i.e., the perfusate went through the aorta, forcing the salt solution through the coronary arteries and out through the sinus. So, eventually, we had a flow that was similar to the in vivo situation. There was a reservoir containing a Ringer-Locke solution, which was oxygenated with 95% oxygen and 5% carbon dioxide. The reservoir fluid was pumped by a Sigma-motor pump through the aorta and coronaries, then allowed to drip back into the reservoir in a recirculating system. When a drug or toxin was put into the system, it was put into the reservoir and recirculated to the heart and back into the reservoir until the toxin or drug was dissipated, either by catabolism or by binding in the heart muscle.

The heart was arranged for recording the contractile force. To do this, a strain gage was attached to the left ventricle to measure an inotropic effect. It is a type of isometric recording, since the strain gage is tied so that any contraction between the two points at which it is attached is transduced into an electrical pulse and recorded. The electrical pulse was also integrated and was used to measure the heart rate.

The rate I will discuss is the ventricular rate, not the sinus rate. Two principal observations were employed to study the effects of the toxin and its interactions with other agents. A pressure transducer was used in the perfusion setup to monitor perfusion pressure. This was used mainly to check perfusion resistance, because the rate of flow into coronaries was approximated at about 1 ml/gm of tissue/min. If, for some reason, the coronaries became

occluded, and the perfusion pressure was not measured, the heart could have become edematous without our knowledge. So, before we did anything to the heart, we discarded all preparations in which perfusion pressure had increased during equilibration. Usually, the perfusion pressure remained constant during the hour of equilibration. All preparations that showed initial dysrhythmia or those that showed a gradual reduction in contractile force or changes in heart rate during equilibration were also discarded. We used only those showing consistent physiological parameters before the drugs were introduced.

The system is inadequate in one very important category: we have to depend on its ability to dissolve the oxygen in the Ringer-Locke solution. It is limited so that the amount of oxygen reaching the tissue is not optimal and certainly is not as good as if we were perfusing with whole blood. The reservoir was kept at 37° to $38^\circ C$.

Our first experiments were performed with the toxin as an agonist. We really wanted to see what it could do when used alone. From previous pharmacology, we knew that it could act as an antagonist. The initial dose employed was arbitrary. We began by estimating that, in our 20-gm mice, we could use 1 MU in 20 ml of fluid. Next, we used about 1/500 MU/ml of perfusing solution to begin, and increased the dose in a logarithmic manner until some effect was observed. The earliest noticeable effect occurred with a dose of 1 MU/ml of the solution. This amount produced a negative inotropic effect: it decreased the force of contraction of the left ventricle. This occurred in about 15 min, and if the solution was recirculated, the effect of the toxin was dissipated, and the contractile force returned to normal values.

To give a quantitative estimate of our cutoff point, we studied several hearts to see how long a heart would survive in this preparation. Without anything being done to the preparation, a change in contractile force of about ±6% occurred for 2 hr. Therefore, if any change produced by the toxin were to be statistically valid, it had to be greater than ±6%. With 1 MU/ml, there was a decrease in contractile force of about 22%. This negative inotropic effect has already been reported for the frog heart. I do not believe it has been reported before in a mammalian myocardial preparation. This effect is not dramatic compared with more potent drugs, but we believe the effect is real. When the dose was increased to 3 MU/ml, there were effects on both contractile force and heart rate. The inotropic effect was frequently masked because of the great variation in effect of this dose in the preparation. In about 10 successful preparations, the effect was distributed, so that in the majority of instances it was positive, but in a few

it was negative. When we added the two to get the mean and compared it with our controls, statistical validity could not be demonstrated at the 5% level. Even though it cannot be proved, we believe there is a positive inotropic effect at this dose of toxin. We recorded a persistent negative chronotropism; i.e., the heart rate decreased, and here we were dealing with a 25% ±2% change. We tried to ascertain some knowledge concerning the origin of this negative effect by pretreating the preparations with atropine. We put 1 μg/ml of atropine into this preparation and perfused the heart for 30 min.with it before the toxin was added. When the toxin was added, the negative chronotropic effect decreased greatly. We were then getting a -4% change, which is well within the variations one can expect in the isolated heart without treatment. We believe this is evidence that the negative chronotropism produced by this dose of toxin is somehow mediated by the intervention of ACh,since it could be blocked readily by a very low concentration of atropine. With the next dose used, 10 MU/ml, dysrhythmias and heart block developed within the first 15 min. By observing first the auricular contractions and then the ventricular contractions,there seemed to be a 2:1 heart block. With dysrhythmia, it is, of course, impossible to make any estimate of inotropism or chronotropism, and the perfusion pressure is also adversely affected. This preparation was observed while it was in the state of dysrhythmia for at least 1 hr, and the dysrhythmia did not reverse itself during that period. We thought this was another effect of the toxin acting on the conducting system in some manner.

Then, 30 MU/ml were used, which produced the same changes (a dysrhythmia) as did 10 MU/ml. But the onset was much more rapid. It was detected in 3 to 5 min by gross observation. We did not use higher concentrations, because, from what we had seen with lower doses, we did not believe any valid measurements of inotropism or chronotropism could be made.

We were aware of the observations of Thesleff on the neuromuscular junction, by which he demonstrated that he could reverse the toxic effect there by using excess calcium. We tried the same experiment because, at the time, we thought we were dealing with a kind of conduction block. First we perfused the heart with 10 MU/ml of toxin until the dysrhythmia developed, then we changed the reservoir, switching to a physiological salt solution that contained twice the normal calcium concentration, and then perfusing as usual. When we did this, the dysrhythmia could be reversed, and the normal rhythm could be restored in 5 min. Thus, we had duplicated the observations of Thesleff with our preparation, and, tentatively, we ascribed the results, using his general hypothesis, to the need for a certain amount of calcium ion for normal

conduction of nervous tissue. These are the observations we made using the toxin as an agonist, and, to summarize, we found three distinct effects: (1) a negative inotropism, (2) a negative chronotropism, and (3) the development of dysrhythmias with increasing concentrations of the toxin. Each response was unrelated to any type of dose-response relationship, because we could not subdivide between 1 and 3 MU, or between 3 and 10 MU to produce graded responses; these are all-or-none observations that could be correlated with different concentrations.

Our next experiments involved the use of the toxin as an antagonist. Such an action is related to the observations made as early as 1927, when an attempt was made to ascertain the mode of action of the toxin on autonomic nerves, which were then known to release ACh. If one separated the vagus nerve to the heart and then stimulated the vagus, bradycardia resulted. If the voltage necessary to produce bradycardia was measured in the normal heart, or in a pretreated animal, the relative reactivity of the nerve-muscle preparation could be obtained. The early investigators demonstrated that, if they intoxicated the animal with botulinum, the voltage needed to produce bradycardia upon vagal stimulation increased with time. So, even then, there was very good evidence that intoxication was related somehow to the release of the vagomimetic substance, or ACh. We extended these observations to an in vitro preparation to see whether the toxin was working as an antagonist, in the classical sense. To do this, a dose of 3 MU/ml was used. It was below the dose that produced dysrhythmia, and it was higher than the dose that we had tried initially. It was an arbitrary choice.

The first agonist we used was choline ethyl ether (CEE), a choice dictated by two considerations. We wanted an ACh-like substance to use in our perfusion apparatus that would not be susceptible to hydrolysis, because we wanted to avoid the use of physostigmine. From previous work with the isolated heart, we knew that if physostigmine were added to the bath, eventually synthesis of ACh by the heart would cause ACh to accumulate so that its typical effect would be manifested, and we would not have a nontreated preparation. CEE is not hydrolyzed significantly. It was chosen, also, because we knew, from other experiments, that, it had no effect on choline metabolism, which is something we were very much concerned with at the time. We did not want to do anything that might interfere with the synthesis of ACh. CEE possesses ACh-like effects at postganglionic cholinergic sites and also autonomic ganglia. One would expect that it would produce such a typical effect of ACh as bradycardia. With no pretreatment, we could produce a decrease in rate of 88% ±8.3% with 30 μg/ml of CEE alone, whereas when we added the toxin,

allowed it to circulate for 30 min, and then added the CEE, the negative inotropic effect was reduced to 19% ±4.6%. This was statistically different from what we had observed before (table 33), indicating that the toxin was exerting an anticholinergic effect at some point. To demonstrate that the CEE was acting at ganglionic and postganglionic sites, hexamethonium was used, which is a typical ganglionic blocking drug at a very high concentration of 100 μg/ml. We could reduce the negative chronotropism of CEE to 46% ±8.8%, again statistically different from the effect of CEE in the absence of hexamethonium. But, there was a considerable amount of negative chronotropism that was not blocked, which we attributed to the postganglionic cholinergic action of CEE. It is also evident statistically that the anticholinergic effect of 3 MU/ml of toxin was considerably greater than that of 100 μg/ml of hexamethonium.

TABLE 33

EFFECTS OF PRETREATMENT WITH VARIOUS ANTAGONISTS ON MAXIMUM NEGATIVE CHRONOTROPIC RESPONSE TO CEE AND McN-A-343 IN CATS

Antagonists and dosages	Chronotropic response	
	CEE	McN-A-343
	%	
None	-88 ±8.3*	-37 ±5.0
Toxin (3 MU/ml)	-19 ±4.6	-32 ±2.8
Hexamethonium (100 μg/ml)	-46 ±8.8	—
Atropine (1.0 μg/ml)	—	-6 ±2.7

* Each value is expressed as percent change from control and represents the mean ±SE for five experiments.

Although it is not indicated in the table, 1 μg/ml of atropine completely inhibits the negative chronotropic effect of CEE.

We had, then, two possible sites of action for the toxin. One was the intraneural ganglia in the myocardium itself, and the second was at postganglionic cholinergic sites. We believed we had demonstrated that CEE was

acting at both places. To dissect the action still further is difficult, using the techniques that we employed. In a classical explanation of the action of the toxin, we would place this action at the postganglionic fiber in the myocardium. In subsequent experiments, more classical ganglion stimulants were used, such as dimethylpiperidinium (DMPP), which has effects only on ganglia and does not have any appreciable effects on postganglionic sites at the concentration used (2 μg/ml). With DMPP, we observed the classical negative chronotropism, which was greatly diminished by 3 MU/ml of the toxin; this led us to believe that the action of the toxin was somewhere between the ganglion and the end of the postganglionic fiber.

There was also the possibility that the toxin was having an atropine-like effect, and we were interested in testing this hypothesis.

Here, we used another agonist, McNeill-A-343 (McN-A-343, table 33). This may seem a strange choice when we could have used muscarine to stimulate the postganglionic site, but, at the time we did the experiments, some Russian investigators had reported that they were able to detect heart ganglia that were adrenergic in that,when they were stimulated, they released, not ACh, but norepinephrine or some other catecholamine at the postganglionic terminal. The compound McN-A-343 is a substance that, in vivo, particularly stimulates autonomic ganglia that release catecholamine at their post-ganglionic terminal. To explore this possibility in its entirety, we used this stimulant. We knew the substance McN-A-343 was a very potent agent on post-ganglionic cholinergic sites. When McN-A-343 was used in the absence of any other treatment, it produced a negative chronotropic effect not nearly as great as had been produced by CEE. When we pretreated in the same way as previously, with 3 MU/ml of toxin, no statistical reduction in the negative chronotropic effect was produced, but the effect of McN-A-343 could very easily be blocked with 1 μg/ml of atropine.

This led us to conclude that if the toxin had any significant atropinelike action and acted at postganglionic cholinergic sites, there was no evidence for it in these experiments. Indeed, there was no evidence for such an action of the toxin on other systems, particularly systems involving isolated smooth-muscle preparations, such as the isolated ileum, etc. We also concluded that, if there were any intramural ganglia in the myocardium that released catecholamine at their postganglionic terminal, they were very few, since the drug produced very little effect that could be attributed to ganglionic stimulation. Indeed, most of its effect must have been mediated at postganglionic sites rather than at ganglionic sites. From these experiments using the toxin as an antagonist, we concluded that it seems to have no effect

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at postganglionic cholinergic sites and that its effect has to be on the intramural ganglia or somewhere along the post ganglionic fiber and in the classical sense. This is perfectly compatible with the explanation that the toxin is acting to interfere with the release of accumulated ACh at the postganglionic cholinergic nerve fiber.

I would also like to report on findings observed in the whole animal without trying to interpret then, because they really defy interpretation. It was quite logical for us to put the heart back into the cat, in a manner of speaking, and see what we could do when we transposed our observations in the isolated heart to the intact animal to see what kind of syndrome could be produced. The experiment was performed on a cat that was artificially respired, with an open chest and the strain gage on the ventricle. The heart rate and the mean arterial pressure were recorded and a noncannulating probe around the ascending aorta was used near its base to measure cardiac output from moment to moment. Obtaining the cardiac output and the mean arterial pressure allowed us to calculate values, such as total peripheral resistance, stroke volume, etc. In the whole animal, we translated the dose used in the isolated heart, using the relationship that there were 10 MU of toxin in 1 ml of the perfusate, and we multiplied that by 1,000 to estimate a dose per kilogram. We used doses from 10,000 to 80,000 MU/kg as infusions in the phosphate buffer over a period of about 10 min. We were, in effect, trying to sustain the blood level of the toxin to see if we could get a persistent pharmacodynamic effect. It was somewhat disappointing in that, although we had hoped to reproduce the cardiac effect we had seen in vitro, we did not succeed in vivo, and we could not observe any dysrhythmia with these doses of toxin.

An interesting observation was the general running down of the peripheral circulation. If the stroke volume was calculated 30 to 40 min after the infusion, there was a progressive decline, which was attributed to a decrease in the venous return from the periphery. Simultaneous with this or immediately following it, a fall in the mean arterial pressure occurred, which progressed during the life of the preparation. Finally, it reached critically low levels before any effect was observed on the myocardium. The contractile force and heart rate were not adversely affected initially, but the peripheral circulation was, somehow. Before death, the animal developed a shocklike condition, in which the peripheral circulation decreased and the venous return became smaller and smaller, until there was not enough circulation through the coronaries to sustain the myocardium. Finally, contractile force was interrupted, and the preparation died. We had to be careful with the cat open-chest preparation to make our observations within 1 or 2 hr;

otherwise, we could not separate the effects of the toxin from normal deterioration of the preparation. Because of this, it was difficult to perform time - of - survival experiments, but we believe that the toxin has an effect on the peripheral circulation that somehow leads to pooling of blood, and ultimately to insufficient return to the heart.

We tried to extend this observation in order to study peripheral resistance in a segment of the arterial supply by perfusing the forelimb of the cat in a manner that maintained a constant flow while allowing the inflow resistance to change. We were hoping to find that the inflow resistance would decrease and that the arteries would dilate as a result of the injection of the toxin. We injected 10,000 MU in a single administration directly into the arterial circulation and allowed it to recirculate in the forelimb preparation for a period of time, and, quite disappointingly, we did not observe anything. When we allowed the toxin-treated blood to go into the whole cat, the animal developed the syndrome I have described, and it deteriorated, but the segment of artery that had been perfused at a constant rate exhibited no change of vascular reactivity. This leaves us with observations that we cannot interpret. We have not been able to transfer our in vitro results to in vivo preparations.

DISCUSSION

Dr. Edmund Bay (CRDL): I would like to question your in vitro experiments. You stated that 1 MU/ml of toxin depressed the isometric contraction. If we record in vivo, simultaneously, the aortic pressure, left intraventricular pressure, and the left ventricular contractile force, the relations among these parameters resemble figure 52. The contractile-force curve and the intraventricular-pressure curve begin to ascend at the same time (A). The contractile-force curve bends sharply at (C) when the aortic valves open. This part of the curve (A-C) can be called the "isometric contraction" of the myocardium. From (C) to (D) is the ejection phase of the systole when the heart muscle is actually shortening. This part of the curve is, therefore, called the isotonic contraction up to point (D) when the aortic valves close again. More than 50% of the total contraction force (A-D) is isometric in vivo. But, this cannot exist in your in vitro experiments, since the left ventricle is empty. I am questioning, therefore, the phrase that you "measured isometric contraction of the heart." Further, I am questioning whether or not you measured contractile force at all, since the heart does not work against any vascular resistance under your experimental conditions. We can talk about amplitude changes during in vitro experiments but not about an isometric contractile force.

The other question concerns your statement that McN-A-343 has a ganglionic action that is excitatory. Do you attribute this effect to the adrenergic property of the compound?

Dr. Rosenblum: I said that in vivo, its effect is associated with ganglia that innervate the adrenergic postganglionic fibers.

Dr. Bay: But, you do not think it has adrenergic effects at the ganglionic sites?

Dr. Rosenblum: The effect is blocked with muscarine rather than with hexamethonium, so that it affects a different locus on the ganglion as does ACh or ACh-like drugs.

Dr. Bay: That is correct.

Aortic pressure

Left intraventricular pressure

Left ventricular contractile force
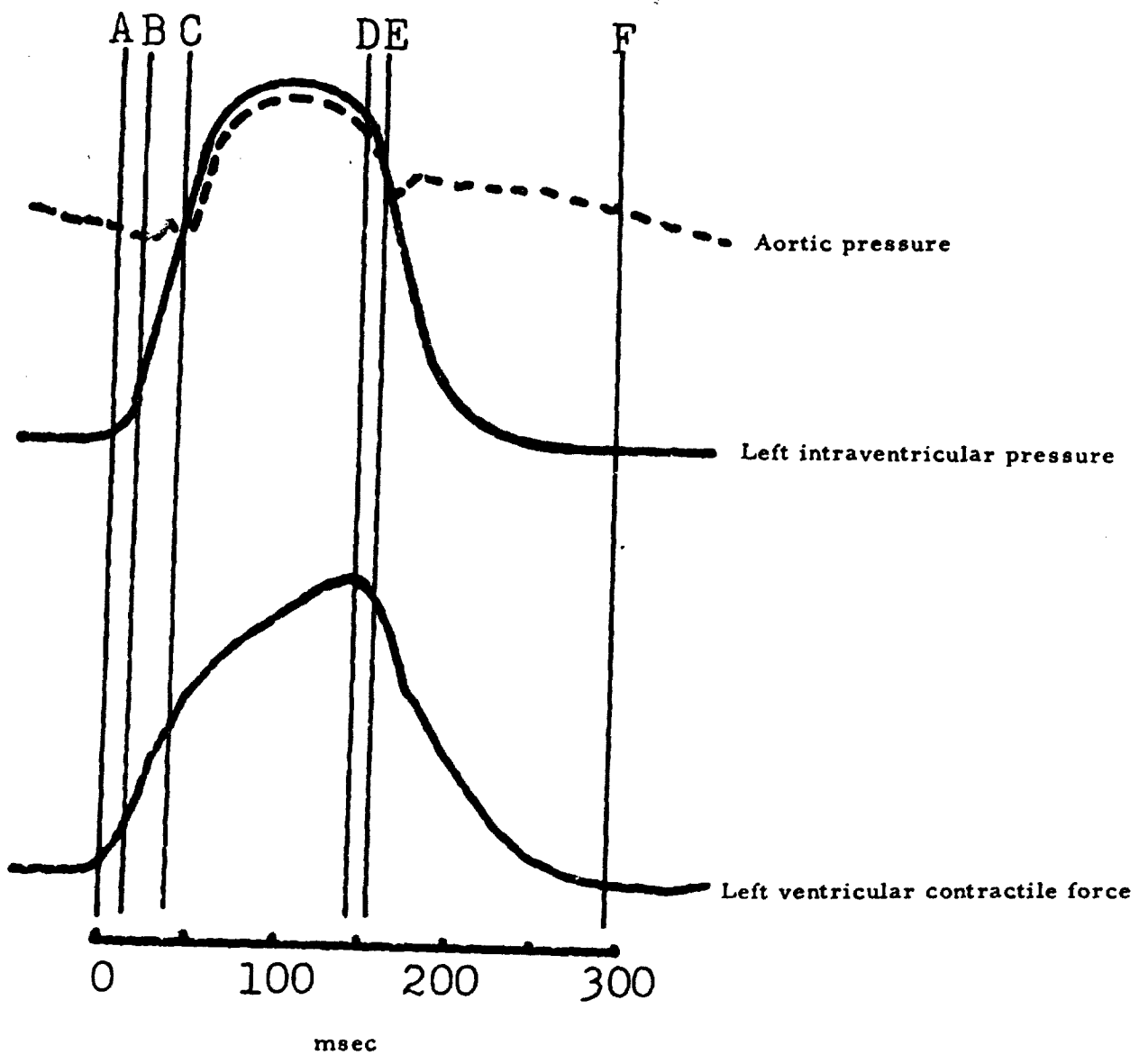
FIGURE 52

DIAGRAM OF THEORETICAL IN VIVO CONTRACTION OF
HEART IN CAT GIVEN 1 MU/ML OF TOXIN

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Dr. Rosenblum: I think, however, it is valid to say that these are isometric in the sense that we have isolated a segment of the left ventricle. It is a strip of left ventricle, essentially, since both points on the transducer are fixed and the only thing that can contract is the ventricular tissue fixed between the two points. They cannot move very far, about 0.001 in. So, I think it is isometric in that sense. I do not think that the heart is doing any work, because the left ventricle is empty; but, I think the contraction measured is isometric.

Dr. Bay: If we accept your explanation, it is very hard to understand that in your in vitro experiments you observed significant cardiac effects using relatively small doses of the toxin, and, on the other hand, in your in vivo experiments you used several doses that were a thousand times larger that did not cause significant cardiac action.

Dr. Rosenblum: I think that is a semantic problem. It cannot shorten in this preparation, except on the order of 0.001 in., because it is fixed between two points. It is as if we had excised the ventricle, fixed one end, and then attached the other end to a strain gage.

Dr. Bay: You cannot talk about isometric contraction during these two time events during intraventricular contraction. You see, from this point to this, the valve is open, and from this point to this, the valve is closed. You cannot talk about force because there is no resistance. It does not work against a resistance.

Dr. Rosenblum: The ventricular muscle certainly does because it has to pull against a fixed point of attachment. It does contract; you have to grant that. It is a contraction without ventricular filling, and since both points are fixed, it is pulling against a resistance and is exerting force.

Dr. Bay: You exposed the heart to 1 MU/ml in in vitro experiments, and there was no cardiac effect, and when you gave iv . 10,000 to 80,000 MU per cat, there was no alteration in cardiac performance by any means. Are they not comparable doses?

Dr. Rosemblum: I like to think that the reason we did not see any effect in vivo is because we did not have as much toxin circulating per milliliter of blood, which is my own explanation. I do not know how much of it was residing in the blood during the experiment. From what I have learned this morning, very little, relatively. Thank you.

EXPERIMENTAL BOTULISM IN THE MONKEY, A CLINICAL-PATHOLOGICAL STUDY (FILM)

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Experimental Medicine Division
CRDL

Our group studied experimental botulism using Type A toxin in monkeys. A syndrome was discovered that resembled one seen in humans after accidental exposures, with a very uniform sequence of events. We used 101 rhesus monkeys while studying the clinical symptoms and, at the same time, we measured the iv and ig LD50's.

The group of monkeys used was healthy and hardy, having been conditioned for 6 mo before arriving here. They were tested for TB and observed closely. After arriving at Edgewood, they were kept under close observation, with further tests, so that only two became ill from parasitic infestation. No illness complicated the experiment, although some parasitic infection was found when autopsies were made on the animals. We also used a group of squirrel monkeys, which were treated similarly. The animals were kept 25 to 50 in gang cages until the beginning of the experiments; then they were separated and confined in individual cages. Before injections of toxin, the animals were observed for gait, aggressiveness, and eye reactions. Individual differences were noted. Blood examinations were made at this time, with sodium and potassium measurements, ChE, etc.

The monkeys were divided into groups of seven, and the toxin furnished by the Basic Toxicology Branch was injected in various doses to obtain the LD50. A bioassay of the toxin was performed simultaneously. . The injections were made with a syringe carrying a three-way stopcock and a reservoir of saline solution as well as the toxin. Entry of the needle into the femoral vein was verified with the saline solution, the injection was made, and the toxin was flushed through with more saline solution.

The following clinical signs were seen after the injections, and they can be seen in the film. The first sign was visual disturbance, noted when animals taken from the cages for tests wandered into furniture. Twenty-four hours after the injections, ptosis of the eyelids was noted. This was followed by muscular weakness, initiated by dropping of the head. Other signs of weakness were a tendency to lean against the cage for support and hanging on the cage by the forelimbs. Finally, the animals simply lay on the floor. The tendon reflexes were hypoactive in the later stages of intoxication.

Respiratory difficulties developed after the skeletal weakness. Breathing in these animals is normally a closed-mouth, costoabdominal process. Yawning and sighing were followed by shallow respiration and what we called a double inspiratory lift. White, frothy, oronasal discharges then appeared. We could not decide whether this was a sign of increased secretion or difficulty in swallowing. Later, eating and drinking ceased, again possibly because of muscular weakness that made chewing and swallowing difficult. A few animals showed signs of a desire to eat by soaking biscuits in water, thus aiding in the eating process. The animals that survived the infection lost the signs of illness in the reverse order, with ptosis of the eyelids being the last sign noted during recovery. In one animal this took 40 days. Muscular strength and activity were restored more quickly. No fever, vomiting, or diarrhea were seen, nor were there changes in pupil size, although the latter has been reported in other studies.

The iv LD50 for rhesus monkeys was about 40 MU/kg (mouse ip LD50). With the ig route, it was about 30,000 MU/kg. In the squirrel monkey, the iv LD50 was 56 MU/kg. The difference in the LD50's of the two species is paralleled by the sharp differences in behavior and in the greater tendency of the squirrel monkeys apparently to cause less death in the colony.

No significant differences appeared in the clinical tests during the experiment. There was a drop in hemoglobin, hematocrit, and erythrocyte count, which may have been caused by frequent blood sampling. Table 34 shows some of these findings and includes clinical laboratory data obtained on monkeys 2 through 7. Seven samples of blood from three monkeys were tested for antibodies by serum neutralization titration. No antibody was found. These samples were from the only survivors of 10 monkeys that received 40 to 50 MU/kg.

All animals that died were examined at autopsy. Significant findings appeared only in the respiratory tract, where areas of atelectasis were found frequently, along with some bronchopneumonia that appeared in animals getting the low doses and that survived longer.

Table 35 shows time to onset of signs and death after iv administration of botulinum toxin to the monkeys.

(At this time, the film, Experimental Botulism in Monkeys, was shown.)

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TABLE 34

CLINICAL LABORATORY DATA OBTAINED ON MONKEYS 2 THROUGH 7

Days from injection	Hematocrit	Hemoglobin	RBC count	WBC count	Differential white count						BUN	Na+	K+	ChE
					Pmn	Band	Lymph	Mono	Baso	Eos				
	%	gm %	×10⁶	×10³							mg %	meq/ml	meq/ml	μmoles
-4	41.0	12.8	5.1	11.25*	26	0	71	0	0	3	19.9	160	5.7	133.6
-3	39.3	12.6	5.1	10.70*	39	0	58	1	0	2	14.8	145	7.7	111.5
3	36.3	11.3	4.2	10.45	40	0	54	2	1	3	24.9	138	4.2	124.1
4	–	–	–	–	–	–	–	–	–	–	21.3	142	4.6	124.4
5	35.8	11.4	5.2	8.75	50	0	46	3	0	1	–	150	4.7	118.7
6**	–	–	–	–	–	–	–	–	–	–	32.9	140	5.0	124.0
9	–	–	–	–	–	–	–	–	–	–	33.1	141	4.8	–
16	–	–	–	–	–	–	–	–	–	–	24.4	137	4.2	118.2

* Control WBC values for these six animals ranged from 7,500 to 16,750.

** Monkeys 2 and 7 died.

TABLE 35

TIME TO ONSET OF SIGNS AND DEATH AFTER IV ADMINISTRATION
OF BOTULINUM TOXIN TO RHESUS MONKEYS

Dose of toxin	Onset of signs, average hours postexposure				Mortality fraction, average hours postexposure
	Ptosis	Muscular weakness	Respiratory distress	Oral and nasal discharges	
MU/kg					
38	65 (32 – 93)	142 (93 – 260)	108 (93 – 140)	127 (93 – 164)	3/6, 199 (127 – 271)
44	55 (30 – 74)	72 (60 – 96)	98 (72 – 120)	94 (73 – 286)	2/6, 120 (119 – 121)
46	31 (22 – 42)	50 (45 – 53)	86 (48 – 147)	89 (86 – 93)	6/6, 130 (53 – 174)
52	26 (13 – 35)	62 (40 – 91)	82 (55 – 109)	82 (61 – 97)	5/6, 274 (70 hr – 40 days)*
55	24 (19 – 31)	55 (41 – 65)	67 (41 – 86)	62 (41 – 89)	5/5, 101 (61 – 154)
55	50 (36 – 57)	84 (66 – 103)	94 (79 – 103)	94 (79 – 124)	6/6, 149 (115 – 185)
65	31 (19 – 54)	43 (36 – 51)	62 (56 – 70)	62 (58 – 70)	5/5, 91 (79 – 116)

* One monkey died at 40 days after a fight with another monkey. If this death were omitted, the average time to death in this group would be 103 hr.

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DISCUSSION

Dr. Sheff (The Pennsylvania Hospital): In reference to the electrolyte figures in table 35, potassium seems to have fallen from 6 or 7 meq/ml in the controls to about 4.5 meq/ml in the experimental animals. Do you consider this significant?

Dr. Streett: No, we do not.

Question: You have never tried to force-feed during the experiment?

Dr. Streett: No.

Dr. Serrone (Albany Medical College): I would like to know if you attempted any blood analysis, perhaps after 14 days, or did you try to find the toxin in the tissues at autopsy? Also, did you notice the narcosis that has been reported after injections?

Dr. Streett: No, we did not.

Dr. Serrone: Have you made any blood analyses for the toxin?

Dr. Streett: No, not from the tissues of these animals. We did save the diaphragm, but it was never analyzed. On some animals receiving higher doses, Dr. Petty analyzed some intercostal muscle, and toxin was found in that.

Dr. Sternberger (CRDL): You have observed a great number of monkeys very carefully and documented them on film, and I think you and your associates have tested additional monkeys since then by injecting them with botulinum toxin. A current progress report states that unconsciousness is associated with injection into monkeys and that this is also a common occurrence in botulism in man. Did you observe unconsciousness in any of your monkeys?

Dr. Streett: No, we did not. This was brought to my attention, and I observed other monkeys. I agree with the idea and possibly attribute it to the toxin used, to the dilution, and to the vehicle that was used. We have not seen any sleepy monkeys in any of those used here. In fact, we would not venture to put a hand in front of any of them.

Dr. Coulston (Albany Medical College): This is a beautiful piece of work, and I want to compliment you highly. I have two comments. First, we would consider a change in the polymorphonuclear-lymphocyte ratio as significant in our laboratory. Second, it is very interesting that these monkeys, as sick as they

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were, tried to eat and drink; at least, the pictures show that. This is most unusual, because when the rhesus monkey becomes sick from a drug or enteritis or anything, it will not drink or eat, and this is a phenomenon that prompts me to ask if there was dehydration or any other factor here.

Dr. Streett: No, not that we could tell.

Mr. Rothberg (CRDL): Was there any attempt to do an antibody titer on the surviving monkeys to see how resistant they were to a challenge?

Dr. Streett: Not the monkey you saw in the film, but we did use a couple of the survivors in another experiment in which we injected much higher doses. We were trying to demonstrate the disappearance of the toxin from the blood. We took a regular control monkey and one that had had an injection over a year ago and saw no difference in the symptoms or the time to death. I would say that this was a hard thing to evaluate. They were given nearly 100 LD50's.

Dr. Tyler (Peter Bent Brigham Hospital): Did you look in the brainstem for infarcted areas or for little blood-vessel lesions with leukocytic accretions that have been reported 30% or 40% of the time in man? There have been small vascular occlusions limited strikingly to the brainstem and the meninges in some patients. Did you look there specifically? Also, you stated that the neurons were normal. About half of the investigators have described changes in neurons and considered that these might be postmortem artifacts. Were any of these present?

Dr. Streett: No, we did not notice any.

PRELIMINARY STUDIES OF LATEX AGGLUTINATION AS A TEST FOR BOTULINUM TOXIN

Dr. Scott V. Covert
Albany Medical College

In an early discussion of our approach to detecting toxin in body fluids or in fractions of tissue homogenates, I suggested that it might be possible to utilize an agglutination reaction for this purpose. Graduate students and colleagues in the department had devised tests of this type using both serum antibodies and soluble antigens adsorbed on latex particles. The procedure had been successful using antigens of Reiter treponemata and antibodies of hemophilus and cryptococcus. We, thus, had accumulated a certain amount of experience in using this technique. We decided to ascertain the feasibility of this technique in both aspects; that is, using the toxin adsorbed to latex for detection of the antibody and subsequently determining the effectiveness of immunizations in group members or using antibody-coated particles for detection of the toxin.

Initially, the detection of toxin was attempted. The early tests were disappointing, because reading the agglutination was difficult, and slide tests were negative. At this time, my supply of antitoxin was expended, and I put this study aside and investigated the reverse procedure; namely, testing for an antibody with toxin-coated particles.

Although no monovalent Type A antiserum was available at that time, I had a commercial Type A—Type B antiserum similar to that used for passive immunization. This serum was used as the test material. Also available was crystalline Type A toxin, which was used for the adsorption to latex.

The toxin as received is made up in NH_4SO_4, which makes a heavily turbid suspension. In order to obtain a clear fluid toxin, 4 ml was dialyzed in the cold with two changes of 0.1 M phosphate buffer at pH 7.4. During dialysis, the volume increased to 6 ml.

Previous experience in other systems indicated that a 1:100 dilution of a suspension of 0.79μ latex particles from a stock having an absorbance of 0.300 was suitable in tests of this type.

The optimum sensitizing dose was determined by sensitizing a standard volume of suspension with 0.01-ml increments of toxin solution, beginning with 0.1 ml and using these various suspensions in reactions with the antiserum diluted in twofold steps to range from 10 to 2,560, based on the

final volume of each tube in the system. The end point was the last tube in the series showing a 2+ reaction, which is defined as a slightly turbid fluid with an appreciable sediment that retains definite clumps on shaking. From this test, the optimum sensitizing dose of this lot of toxin was determined to be 0.12 ml for each 5 ml of suspension of latex particles. Ten minutes were allowed for sensitization, which was performed just prior to making a test.

The test includes the following controls:

1. Saline plus sensitized particles

2. Lowest dilution of serum on the test plus unsensitized particles

3. Normal serum of the species plus sensitized particles

4. Normal serum plus unsensitized particles

5. When testing unknown sera, a control of positive serum with both sensitized and unsensitized particles.

Tubes are incubated at 37°C for 30 min, and a preliminary reading is followed by incubation at 4°C overnight. Usually, there is a two-tube difference in reading after overnight incubation.

Two alternate procedures will be investigated: (1) incubation at 37°C throughout and (2) shaking for 15 min followed by centrifugation to determine whether the test can be read earlier. Although time is not an important factor in this test, it may give indications as to the method to be followed in the reverse agglutination.

A completed test using the reference AB antiserum is shown in figure 53. Figure 54 shows tubes after they have been tapped.

After the details of the test had been established, a check for cross reactions was investigated. Diphtheria and tetanus antitoxin in this system gave no agglutination. The reaction with normal horse serum was negative. I now have a supply of monovalent Type A antiserum, and, when sera for Types B and E become available, these will be checked.

During our immunizations of personnel, blood was drawn before the first injection and just before each booster was given. These sera are available for testing. At this point, I tested my own serum and obtained a titer of 160. As a normal control, blood from the nonimmunized physician who inoculated the group was used. This serum also showed agglutination. Neither serum was inactivated.

FIGURE 53

COMPLETED LATEX-AGGLUTINATION TEST USING
TYPE A–TYPE B ANTISERUM

FIGURE 54

LATEX-AGGLUTINATION TEST TUBES AFTER TAPPING

To investigate this nonspecific reaction, sera submitted to the hospital's diagnostic laboratory for various tests were used as normals. The first group of four consisted of one submitted for febrile agglutinins (negative). The others were submitted for rheumatoid factor (RA) (negative). The RA sera had not been inactivated; the other had. The febrile serum gave no titer at all, but a sediment was present that resuspended easily. All of the RA's gave a titer of 160. This appeared to be true agglutination, since clumps remained after vigorous tapping. The controls of serum plus unsensitized particles were negative.

These sera were reinvestigated after inactivation. The febrile serum reacted as before. The RA sera showed a button of sediment in the bottom in tubes 20 through 160. When tapped, these buttons dispersed to give uniform suspension without clumps. Positive controls gave good clumps on tapping. In two preliminary readings, agglutination of the positive control was complete at the end of the 37°C half-hour period, whereas, at the end of 3 hr at 4°C, no change was apparent in the test series. No further readings were made until 18 hr later, when the above data were recorded. The appearance of the sedimentation of the RA's and the flocculation of the positives were quite different. In addition, after tapping, the positives reverted to their original appearance in a short time; the RA's remained uniformly suspended at 3 hr.

To investigate further, new sera from the hospital laboratory were secured, and four were tested. These consisted of two negative febriles and two positive RA's. They were inactivated and tested as before. These sera again showed a button of sediment through tube 160, but this resuspended easily on tapping. Thus, the apparent agglutination in the original RA serum was no longer seen when the serum was inactivated.

Normal horse sera, used as a control and tested in a dilution series, have not exhibited the button sediment seen in human sera. The result appears to be confined to human sera.

The dialyzed toxin we have discovered remains stable for at least 15 days, since it was tested during this period against the reference serum on eight occasions with no change in end point appearing. If toxoiding or molecular change occurs, it does not affect adsorption to the latex.

Next, we plan to test the sera of our personnel and a series of negative sera submitted as premaritals. This population, we suspect, will represent a younger and, most certainly, healthier group than those tested thus far.

DISCUSSION

Mr. Fleisher (CRDL): It might be of some interest for Dr. Covert to test the immunological potency of the staff antibodies against the subunits that were discussed yesterday to determine, on a comparative basis, whether the same immunological properties exist.

Dr. Covert: If these units become available, this would certainly be one of the tests we would attempt using this system of agglutination. We have done some precipitin tests, and I think that the agglutination test is actually more sensitive than the precipitin test.

Mr. Fleisher: I think Dr. King might have a comment on this, because the IITRI group did some of the work in a recent contract. Dr. King, would you care to comment on this please?

Dr. King (IITRI): Our agglutination tests were not made with latex but with PAB-Cellex, which is cellulose that has been modified by the addition of p-aminobenzyl groups. We were able to diazotize both toxin and antitoxin to the Cellex. Since some difficulty was experienced with the commercial antitoxin, crystalline toxin was converted to the toxoid and used to produce antitoxin in rabbits. Good reactions were obtained with solutions containing as low as 0.1 µg of crystalline toxin. In the direct system, the antitoxin was diazotized to Cellex and incubated with test solutions of the toxin to obtain agglutination. When we realized the possibility of small subunits being present in some of our toxin preparations, however, the indirect system was used to ensure a reaction.

Mr. Fleisher: Dr. King, it seems clear just what your experience was with this, but I am not sure I follow this.

Dr. King: In this instance, the toxin is diazotized to the Cellex. Dilutions of the test solution are incubated with a standard quantity of the antitoxin and then reacted with the Cellex toxin. The quantity of antitoxin remaining varies according to the dilution of the test solution. The point at which agglutination begins is a measure of the amount of toxin present and is independent of the size of the units.

Dr. Sternberger (CRDL): Why did you use Cellex instead of bistetraazotized formalinized erythrocytes? The sensitivity with erythrocytes would be several thousand times greater than it would be theoretically with the Cellex, which is of variable particle size.

<u>Dr. King</u>: The Cellex was a little more sensitive than the erythrocytes, and, actually, we were able to see the agglutination reaction very readily.

<u>Dr. Sternberger</u>: With the Cellex, you have the additional problem of having a very large amount of diazo-type material, and, as we heard yesterday from your group, tyrosine is very sensitive to substitution. It would be very surprising if you did not inactivate the toxin by this procedure. I would, however, like to commend Dr. Covert on his very significant work because I think it is a very simple test, and there has not been much successful work on simple tests. A simple test is very important here, because the toxin is rather unstable, and it is very important to find a test that is more reproducible than other in vitro tests.

Lamanna has described the disturbance observed in the precipitin reaction with an excess of antigen that will not let him observe soluble complexes because the excess of antigen will cause nonspecific precipitation. He ascribed this to the hemagglutinating principle. I wonder if he used inactivated serum and if your effect of inactivation or lack of inactivation could be related to this nonspecific factor of the hemagglutinating principle? I think, perhaps, some answers can be derived in this area if you perform your latex-agglutination test in the presence of excess toxin. In an immunologic reaction, an excess of toxin would not lead to agglutination, whereas a nonspecific reaction would. Did you have a chance to study the effect of inactivation under these circumstances?

<u>Dr. Covert</u>: We did not do that, but I think it should be done.

<u>Dr. Crisley (Taft Laboratories)</u>: Have you attempted to measure the relative sensitivity of your latex test, relative to, say, the mouse test?

<u>Dr. Covert</u>: No; however, this is one we do contemplate. Actually, this is preliminary, and most of the work has been done within the last few weeks. We hope to do some of these tests, and this is one we have in mind.

<u>Dr. Petty (Maryland Medical-Legal Foundation, Inc.)</u>: I am curious to know what your serum titration of antitoxin actually was at the time you drew the blood for this latex test.

<u>Dr. Covert</u>: The serum titration?

<u>Dr. Petty</u>: Yes. Did you know how much antitoxin was circulating in your serum at that time?

Dr. Covert: No. There have been no mouse tests nor precipitin tests done. They should be done.

Dr. Sim (CRDL): I have similar questions to ask. What were the immunization schedules, and at what times were titers determined? The importance of high versus low postimmunization titers raises further questions. Your finding of possible alterations due to febrile illnesses is important.

Dr. Covert: I can tell you. Our protocol was an original inoculation with a booster 3 mo later. The serum I checked was the serum drawn just prior to the first booster. Two boosters have been given since then, and I should test these sera to see if the titer is actually rising. This was what we were trying to determine in the first place.

Mr. Fleisher: I would like to call the attention of the Albany group to similar work performed at Stanford about 2 yr ago in this same direction. They found that the tests varied in sensitivity, depending on the source of antibodies. Chicken red cells, appropriately sensitized, were most sensitive for them, and I thought that Jim Richards might like to comment on this, since he was the contract officer for this group. Jim, do you recall the time involved? I think they could get a reaction and complete the test in 30 min, contrasted to the overnight period.

Mr. Richards (CRDL): Yes; by the method we used, we could get a complete test in 30 min. We developed our test in indented plates.

Dr. Coulston (Albany Medical College): I think Dr. Covert could speak on this better, but, actually, the test he described in its entirety is a quantitative test. Qualitatively, you can see agglutination in a matter of 10 min, and you can tell if there are antibodies present or not. I wanted to make this point: we can do it speedily too, but the degree of getting it out to two more tubes is why we went to a greater extent.

Mr. Richards: We developed it in a dilute solution as well, so I think the objection of quantitativeness is not valid in this instance.

Mr. Sass (CRDL): First, I would like to say, Dr. Covert, that using the latex is in the line of what we were looking for with Dr. King at IITRI. What we were looking for was a reagent that could be stored; in other words, another chemical reagent for analysis, to be quantitative and specific. We had not intended this to be used just as a biological fluid, but in toxin cultures and so forth, so that we could distinguish between the toxic and nontoxic moieties.

What we learned was that it is quite feasible; the problem is that the Cellex-p-aminobenzoate was not uniform from batch to batch. In other words, some batches had more active centers than others, but it looked as if it were a feasible system within batches.

Dr. Covert: I think the latex is really a very good vehicle for this, because it is a fairly uniform thing, and I have no doubt that we can also put the antibody on these particles.

Question: What was the approximate concentration used? Were you diluting the original material?

Dr. Covert: The approximate concentration was measured in milliliters, and we used 0.12 to 5 ml of suspension. I can figure it out for you. I think we have the number of milligrams, at least of the antigen nitrogen for this material.

Dr. Sternberger: I think latex is obtainable now in very uniform characteristics, because it is made uniform for other purposes. Cellex may introduce another problem, however. If you couple diazotized Cellex, the reaction between the solid diazonium groups and the liquid toxin is somewhat time-consuming, and coupling may be incomplete. I think, therefore, if this procedure is used, it will be absolutely essential to add β-naphthol afterwards to make sure that all diazonium groups are being utilized. This process will take several days for completion, as shown by our own experience; when the Cellex is fairly uniform, it can be obtained with certain precautions. But, when you do all this, the conditions of coupling require a fairly alkaline pH to be successful. If you do not use fairly alkaline media, you end up with unreacted diazonium groups that are troublesome later. If you have free diazonium groups in your material, you can get a great deal of nonspecific agglutination. On the other hand, if you try to use alkaline conditions for coupling, you invite denaturation of the botulinum toxin.

Dr. King: We actually followed the method of Campbell,* in which β-naphthol is used to remove the unreacted groups. By utilizing this procedure, we obtained reproducible results with a given batch of Cellex. If we had obtained Cellex containing more p-aminobenzyl groups, our test would have been much more sensitive than it actually was.

Dr. Sternberger: Was there any inhibition by excess toxin?

Dr. King: That was part of the indirect method that we utilized.

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* Malley, A., and Campbell, D. H. J. Am. Chem. Soc. 85, 487 (1963).

SESSION IV

29 June 1965

Chairman: Dr. J. H. Wills
Chief, Physiology Division, CRDL

RECENT RESULTS FROM ILLINOIS INSTITUTE OF TECHNOLOGY

Dr. W. H. Riesen

Illinois Institute of Technology Research Institute

Dr. Wills (CRDL): Dr. Riesen has just received some new information that he would like to pass on to the conference, so I will invite him to start the session.

Dr. Riesen: We have attempted in the last few days at IITRI to repeat the work of Gerwing, Dolman, and Bains* with a Cl. botulinum Type A culture broth that was obtained through the courtesy of Dr. E. J. Schantz. The results of this work will necessarily be preliminary and will be subject to confirmation in repeat experiments. Dr. G. Sumyk at IITRI has been working actively on this problem, and Dr. M. King and I have been in communication with him on the recent results.

In the Canadian work, the Dolman strain of Type A toxin was cultured to a maximal titer and the cells were centrifuged. At a titer of 100,000 to 300,000 mouse MLD units/ml, they adjusted the ammonium sulfate concentration to 50% (v/v) saturation, dissolved the precipitate in 0.01 M citric acid buffer, and passed the solution through a DEAE cellulose column. They obtained four peaks in the effluent with virtually all the toxicity in the frontal peak. It was found homogeneous when examined by velocity sedimentation, electrophoresis, and diffusion. They calculated a molecular weight of 12,200 from the S_{ob} coefficient and the diffusion constant, making certain assumptions regarding density.

The broth of the Detrick Type A strain has a considerably higher toxin titer than was obtained by the Canadian investigators with their strain. In our work, the cell-free broth was adjusted to 50% ammonium sulfate, and the precipitate dissolved in citrate and passed through a DEAE cellulose column in the same manner as Gerwing. We obtained one peak instead of four. The peak had toxicity and was not further characterized.

The procedure was then modified. A gradient elution decreasing in pH was set up. Two peaks were obtained: one, the frontal, was toxic and was centrifuged in a synthetic boundary cell at top speed for several hours. No fast-moving component was found corresponding to the 900,000-molecular-weight moiety. Instead, one very-slow-moving major peak was found. It appears to be very low in molecular weight and has high toxicity in terms of

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* Gerwing, Julia, Dolman, C. E., and Bains, H. S. J. Bacteriol. **89**, 1383 (1965).

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nitrogen. Drs. Schantz and Spero at Fort Detrick have looked at the culture filtrates of this strain, grown in sacks. Very high titers were obtained under these conditions. They looked at the culture broth in the ultracentrifuge and found only a fast-moving boundary, analogous to a molecular weight of 900,000. No toxicity was found in the supernatant of this material. This would suggest the absence of any predominantly small molecular species in the broth after release of the toxin in cell lysis.

The inference is that the toxin of this strain when first produced in the culture medium has a high molecular weight. It is possible that the ammonium sulfate depolymerizes the material in fractionation.

PRODUCTION OF HUMAN ANTIBOTULINUM SERUM

Dr. Charles S. Petty

Maryland Medical-Legal Foundation, Inc.

In 1910, Leuchs* first produced botulinum antitoxin in horses, and the product that he was responsible for is essentially the same that has been used for therapy of botulism ever since. In 1940, Lederle** began the commercial production in this country of Types A and B antitoxin, again produced in horses.

In 1924, the first toxoid for botulinum was produced.† In 1934, a Russian, Velikanov, †† began using toxoid experimentally. He immunized humans. By the late 1930's and through the 1940's, Types C and D botulinum toxoid were used extensively to immunize certain valuable animals against botulism. This included sheep, cattle, and, later, mink. In 1962, Fiock and coworkers‡ at Fort Detrick reported some studies that they had performed with bivalent toxoid and reported the antitoxin titers that were elicited in volunteers. Figure 55 is from their work. Various immunization procedures were tried using Types A and B toxoids. Several immunization sequences are on the chart: one is for 0, 2, 4, and 6 wk, another for 0 and 8 wk, and another for 0 and 10 wk, etc. Results are given both in A titer,

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* Leuchs, J. Beiträge Zur Kenntnis des Toxins und Antitoxins des Bacillus Botulinus. Ztschr. Hyg. u. Infektionskrank 65, 55-84 (1910).

** Cooper, M. S. Public Health Service Publication 999-FP-1. Antitoxins to C. Botulinum. pp 147-164. 1964.

† Weinberg, M., and Goy, P. Recherches sur la Toxin Botulinique. Compt. Rend. Soc. Biol. 90, 269-271 (1924); Weinberg, M., and Goy, P. De l'Anatoxine Botulinique. Formolée et Iodée. Ibid. 91, 1140, 1141 (1924); Weinberg, M., and Goy, P. Emploi de l'Anatoxine dans la preparation de Sérum Antibotulinique. Ibid. 92, 564, 565 (1924).

†† Velikanov, I. M. Experimental Immunization of Man Against Botulism. Klin. Med. 12, 1802-1906. (1934).

‡ Fiock, M. A., Devine, L. F., Gearinger, N. F., Duff, J. T., Wright, G. G., and Kadull, P. J. Studies on Immunity to Toxins of Clostridium Botulinum. VIII. Immunological Response of Man to Purified Bivalent AB Botulinum Toxoid. J. Immunol. 88, 277-283 (1962).

the cross-hatched bar, and B titer, the solid bar, at various intervals following the immunization of these volunteers. In all probability, the 0-, 2-, 10-wk sequence produced the highest number of "satisfactory" antitoxin levels. A satisfactory level indicated that there was at least a detectable quantity of antitoxin of the type in question; in this study, these were Types A and B only.

Later, Fiock, Cardella, and Gearinger* at Fort Detrick published some information on the titers resulting from immunization with pentavalent toxoid.

The data listed below give the antibody titers in Porton units/ml. I have selected, from their work, only three of the five types (A, B, and E) because I am concerned primarily only with those three types.

		12 wk	Antibody titers	
			52 wk	8 wk postbooster
			Porton units/ml	
Type A	Mean	0.07	< 0.02	1.3
	Median	0.05	< 0.02	0.6
	% Measurable	65	0	100
Type B	Mean	0.03	< 0.005	0.2
	Median	0.03	< 0.005	0.1
	% Measurable	82	6	94
Type E	Mean	0.05	0.007	2.1
	Median	0.03	0.008	0.8
	% Measurable	94	94	100

After 12 and 52 wk and then 8 wk postbooster, a certain percentage of individuals developed measurable titers: 65% with A, 82% with B, and 94% with E. When a booster was administered to these individuals and blood collected 2 or 3 wk following the injection, the percentage of measurable titers rose to nearly 100% for each of the three types under consideration.

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* Fiock, M. A., Cardella, M. A., and Gearinger, N. F. Studies on Immunity to Toxins of Clostridium Botulinum. IX. Immunologic Response of Man to Purified Pentavalent A, B, C, D, E Botulinum Toxoid. J. Immunol. 90, 697-702 (1963); Cardella, M. A. Public Health Service Publication 999-FP-1, 113-130. Botulinum Toxoids. 1964.
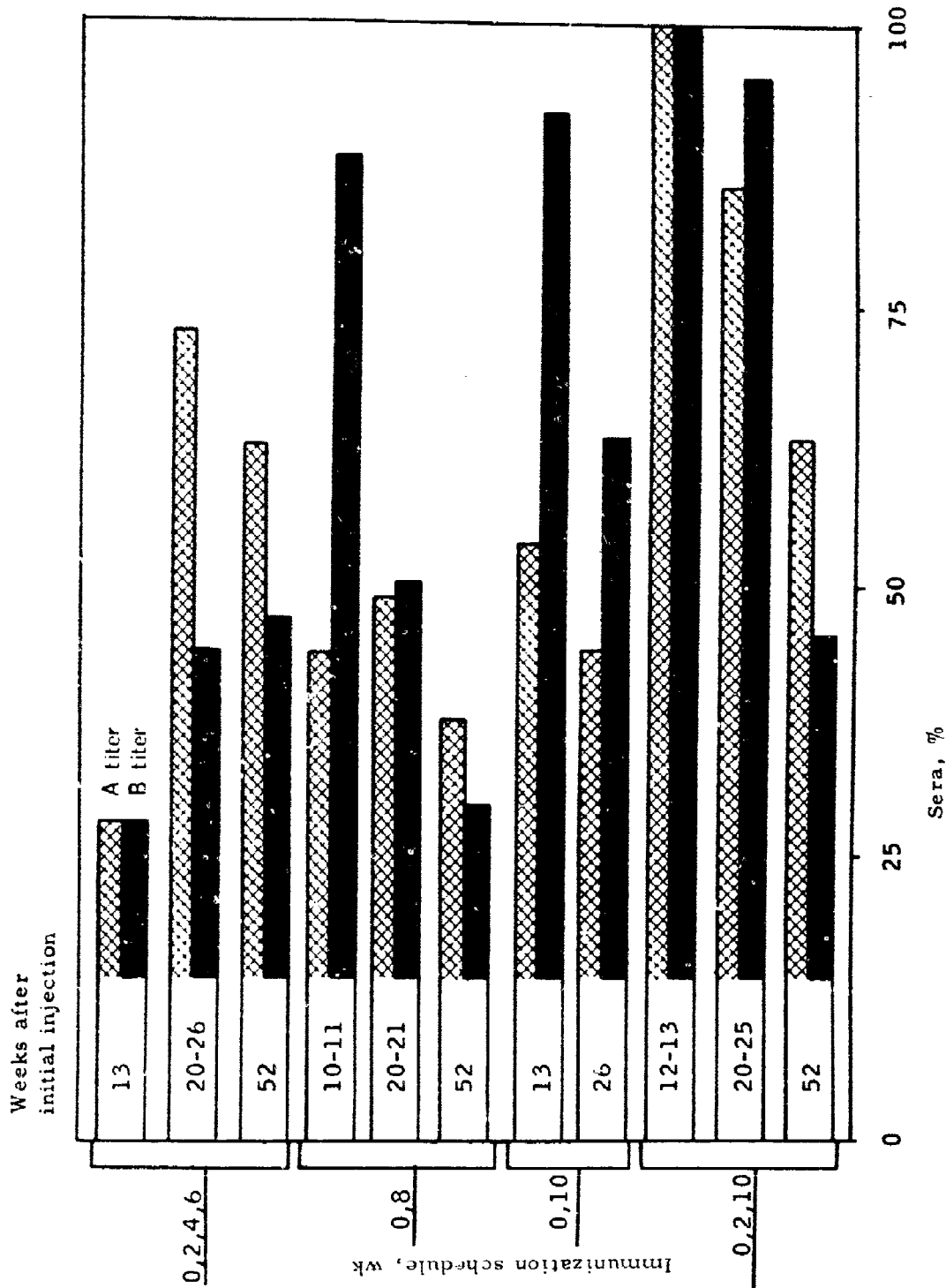
FIGURE 55

PERCENT OF SERA WITH SATISFACTORY ANTITOXIN LEVELS

The pentavalent toxoid, with which most of you are familiar, is a product that was produced on contract by Parke, Davis, and Co. at the instigation of Fort Detrick. It has been used widely. As far as I know, about 2,000 people have been immunized, or immunization has been attempted in them with this product.

A year and a half ago, the gentlemen at Fort Detrick,Dr. Glassman, Dr. Kadull, Mr. Cardella, and others, were kind enough to allow me to come into their establishment and to attempt plasmapheresis on several individuals who had received long-term injections of the pentavalent, and in some instances pentavalent plus univalent, toxoid. It was our intent to take from these people serum that could then be examined for content of antitoxin and also to see if we could obtain a large quantity of serum by a double plasmapheresis system. In addition, we wished to see whether or not it would be feasible to set up an injection plan in humans whereby the human might serve as the production animal for botulinum antitoxin. Several individuals were bled and plasma-pheresed in February 1964.

The A, B, and E antitoxin titers were worked out by Fort Detrick and in our laboratory for comparison. Table 36 shows that the figures agree well. The E antitoxin was not titered in our laboratory, only at Fort Detrick. The average titer of the seven individuals is about 12 Porton units/ml of A, nearly 0.5 Porton unit of B, and about 5 Porton units of E.

TABLE 36

ANTITOXIN TITERS IN VOLUNTEERS

Plasma No.	Antitoxin titers				
	Type A		Type B		Type E
	Detrick	Med-Leg Lab	Detrick	Med-Leg Lab	Detrick
	Porton units/ml				
P1	12.80	10.4	<0.01	>0.01	2.0
P3	12.80	10.5	0.80	0.35·	0.5
P4	12.80	9.7	0.95	0.4	2.0
P5	3.20	2.8	0.20	0.08	0.2
P6	20.5	38.4	0.08	0.3	>12.80
P7	12.80	10.6	0.80	0.53	8.0
P8	15.46	9.0	0.95	0.65	8.0
Average	12.7	13.1	0.54	0.33	4.8

Two of the men were immunized not only with the pentavalent toxoid, but they also received some univalent toxoid. If you eliminate them, there are five people who had received four or five injections of pentavalent toxoid. Table 37 shows the antitoxin levels of these persons. These were most encouraging results. Having done this, and having found that we could take about 500 ml of serum from each man at one sitting with the double plasmapheresis technique, we began a system of immunization of our own volunteers.

TABLE 37

ANTITOXIN TITERS IN VOLUNTEERS RECEIVING ONLY PENTAVALENT TOXOID

Plasma No.	No. of injections	Antitoxin titers			Time post-booster
		Type A	Type B	Type E	
		Porton units/ml			days
P3	4	10.5	0.35	0.5	24
P4	5	9.7	0.4	2.0	40
P5	4	2.8	0.08	0.2	46
P6	5	38.4	0.3	12.8	29
P7	5	10.8	0.53	8.0	22
	Average	14.5	0.33	4.7	

The immunization schedule that was followed by the Detrick workers varied from one group to another, and, lacking any specific guide as to how one should go about this (and it seemed to be a purely arbitrary arrangement), we worked up a system of giving the pentavalent toxoid that would be satisfactory for our own time schedule, as follows:

"Volunteer" injection schedule (weeks)

$$0 - 2* - 4 - 16 - 28 - 2^{B*} - ?^{B} - 2^{P}$$
$$(12)$$

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* Blood samples.

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Two groups of volunteers were immunized at 0, 2, 4, and 16 wk, and we are now at about the 28th wk with our first group. We took blood from them for a control antitoxin titer at the 2-wk immunization period. It was not practical for various reasons to take it at the initial time of injection. We plan to give a booster about 2 wk after the 28-wk injection, sampling blood at that time and determining the antitoxin level. Following this, it is planned to bleed these people once again a little later, to see what the dropoff in antitoxin titer is, and then give them another booster about 2 wk later and then to bleed them. Possibly, this next step may be omitted entirely. That is why there is a question mark in the schedule.

So far we have been able to adhere closely to our schedule, as shown below. We have two groups, about 50 people in one and 35 in the other. Our 2-wk injection on them was done on 14.69 days average, with a range of 13 to 22 days.

Group I			Group II		
Weeks	Days		Weeks	Days	
0	0		0	0	
2	14.69	(13 - 22)	2	14.25	(13 - 19)
4	14.15	(13 - 19)	4	14.02	(13 - 16)
12	85.08	(75 - 115)			

The ultimate object of this project is to get a product that can be used to treat the acutely poisoned individual. It would be entirely feasible to use this as serum (or as plasma). Upon consideration of the amount that might be necessary, it would seem better to concentrate the active material, if it can be concentrated, and to use it as gamma globulin.

There is the problem of the choice of individuals to be used. We intend, on our first titering, to eliminate those who are poor producers of antitoxin. We will probably have to do some juggling with the "volunteers" to determine who should and should not be included, because, in all probability, one individual will not have a high level of antitoxin for each of the three; i. e., the A, B, and E antitoxins. Our current plan is to produce 10-ml vials of gamma globulin. About 250 ml of plasma should give 1 gm of gamma globulin, and the 10-ml vials will therefore contain approximately 2 gm of this. The output of one man at one sitting, 500 ml of plasma, should give us one 10-ml vial or 2 gm of gamma globulin. Included in this 500 ml of plasma there should be approximately 100 ml of anticoagulant citrate solution, so we are actually

dealing with about 400 ml of pure plasma. If we use the level we found in our original volunteers, about 14.5 Porton units of A, 0.33 of B, and 4.7 of E, by some adroit mathematics we come out to about 58,000,000 units of Type A toxin that should be completely counteracted by this amount of plasma, approximately 1,300,000 of B, and 19,000,000 of E. We do not know, of course, that all the active antibody components of the blood will be included in the gamma globulin. If we take a safe estimate, say 50% (based on work previously done at Lederle* where 40% to 50% of the active material was found in the separated fractions), one 10-ml unit of gamma globulin should be able to neutralize about 29,000,000 units of A, 660,000 units of B, and 9,500,000 units of E toxins. If this is true, one 10-ml vial, or perhaps two, should be a satisfactory dose in the average case to give a therapeutic level of antitoxin. The data are illustrated below.

~250 ml of plasma = 1 gm of gamma globulin

10-ml vials (16.5%) = ~2 gm of gamma globulin

500 ml of plasma = 2 gm of gamma globulin
(less 100 ml of anticoagulant)

	Type A	Type B	Type E
400 ml of plasma =	14.5	0.33	4.7
	5,800	132	1,880
	10,000	10,000	1,000
	58,000,000	1,300,000	19,000,000
50% =	29,000,000	660,000	9,500,000

Now, why worry about human antibodies? Horse antibodies are available for Types A, B, and E, and also probably for Type F in at least some countries. There are certain advantages to the human product—first, speed; that is, speed of treatment. One would no longer have to wait to see whether one were dealing with Types A, B, or E, or for that matter Type C or D. There is broad coverage; that is, one would not have to worry about getting a specific therapeutic serum or material to treat various types of botulism. Next, and I think most important, is the lack of reaction. There is no blood-group problem that one might run into by using serum as

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* Cooper, M. S. Public Health Service Publication 999-FP-1. Antitoxins to C. Botulinum. pp 147-164. 1964.

it comes out of the donor. Then there is a very small chance of getting
homologous serum hepatitis with this product. Millions of units of gamma
globulin have been used in this country for treatment and prophylaxis without
any appreciable number of cases of homologous serum hepatitis.* Then, all
of the therapeutic serum now available is produced in horses (rarely in rabbits).
Various writers** estimated that between 3% and 30% of the individuals receiv-
ing prophylactic or therapeutic treatment for various diseases with horse-
serum material either have been affected with serum sickness or have been
sensitized to horse serum. Several of the botulism cases that have occurred
in the last several years in this country have been associated with serum sick-
ness. I believe Dr. Tyler will affirm this.

There is also ease of administration to be considered. We are
dealing with small dose units, not with 250 ml of plasma. Further, gamma
globulin is not given iv, but by im injection. The absorption from a muscular
site is almost as rapid as an iv injection. Referring to experience with human
antitetanus serum, it is more effective unit for unit than is the horse-serum
antitetanus material. Further, although not particularly applicable to botulinum,
there is a longer half life in the body of human gamma globulin than heterologous
gamma globulin, regardless of the animal that produces it. Therefore, for the
reasons given below, we want a stock of human antiserum for treatment of
botulism.

Advantages

Speed	Ease of administration
No typing necessary	Small dosage units
	Not intravenous
Broad spectrum	
All types covered	

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* Communicable Disease Center. Hepatitis Surveillance Report 22. p 32.
  U.S. Department of Health, Education, and Welfare, Public Health
  Service. March 31, 1965.

** Filler, R. M., and Ellerbeck, W. Tetanus Prophylaxis. J. Am. Med. As-
   soc. 174, 1-4 (1960); Laurent, L. J. M., and Parish, H. J. Serum
   Reactions and Serum Sensitivity Tests. Brit. Med. J. 1, 1294-1297
   (1952); Moynihan, N. H. Serum Sickness and Local Reactions in Tetanus
   Prophylaxis; A Study of 400 Cases. Lancet 2, 264-266 (1955); Skudder,
   P. A., and McCarroll, J. R. Current Status of Tetanus Control.
   J. Am. Med. Assoc. 188, 625-627 (1964).

Advantages (contd)

Lack of reaction	Good potency
No horse serum	More effective, unit for unit
Low potential for homologous	Longer half life in vivo
serum hepatitis	

No blood-group problem

There are some drawbacks to the production of this human anti-botulism material:

Disadvantages

Expense

Time to build effective donor pool

Peripatetic donor pool

FDA requirements

There is expense involved. We are paying our "volunteers" to go through the series of immunizations, to produce blood in small quantities for titering, and, finally, to lie on the blood-donor table for as long as necessary to perform a plasmapheresis. Second, there is a long time involved in building an effective donor pool. You cannot immunize these people overnight or even in 6 mo. It requires time and expense to discard those who are not good producers. Third, the group will prove to be peripatetic. We have lost 3 of the 85 we started with and may lose more, because people are mobile. We cannot use medical students because they disperse after graduation. We cannot use college students because they are usually not 21 yr of age and are legally infants, not able to do this on their own; parental permission is hard to come by. Fourth, one of the problems we are now up against (with apologies to Dr. Hansen, if he is here) is the FDA regulations. These regulations, which were set up to avoid harmful or therapeutically ineffective drugs, were designed for diseases much more common than botulism, which occurs in this country only 15 or 20 times a year. Therefore, the stringent regulations are difficult to fulfill when dealing with a very rare disease.

Now a word of explanation of how we got into this. I am a pathologist, not an immunologist and certainly not a manufacturing chemist, although I am a pharmacist. The mission of this contract was to find out how much botulinum toxin was lethal for the human. Therefore, we started at the obvious beginning, by examining botulism victims, hoping to find cases

where we could quantitate the amount of food and infected or contaminated material taken in and find out what happened to the individual with a known ingestion. Frankly, I got tired of being about 15th in line on these cases. Following the Detroit catastrophe, in which two people died as a result of eating spoiled tuna fish, as soon as the cry of botulism is raised in this country, large forces are put into play—the FDA, the State Health Department and the County Health Department, Fort Detrick, and ourselves; all these agencies become interested in the case, and there is no good chance of getting useful information simply because the patient's chart is in the hands of someone else most of the time. The patient is almost always taken care of by tracheotomy, he is receiving all sorts of injections with botulinum antitoxin, he is being given antibiotics, and he may find himself involved in all sorts of manipulative affairs to keep his respiration in good shape and to prevent pneumonia. As a result, it is very difficult to get information from the patient.

Now it is my plan, if the production of human antitoxin succeeds, to play "immunologic blackmail;" it is my intention to go to the bedside with the material in my pocket and say, "Now look, if you want this material (which we have every reason to believe is therapeutically effective and will not give your patient horse-serum sensitivity) we will be delighted to have you use this, if you let us sit at the bedside along with you. We don't want to report your case in the literature; we don't want to take it out of your hands, but here it is if you play ball with us." We hope this type of "blackmail" will be effective. It was with the organic phosphate problem as some of you may know. I was involved in that for a while, and it was very nice to have the therapeutic agent, the method of diagnosis, and the method of prophylaxis for presickness detection of impending disaster available. I was able to get information that I trust was useful to this organization and to others. That is the reason for getting into this business; this is our plan; these are our hopes. We have every reason to believe that by the beginning of 1966 we will have a therapeutically effective material that is easily used and that will substitute for the material that has now been available for a half century.

DISCUSSION

Dr. Serrone (Albany Medical College): I would like to know if you get any reaction to the toxoid by im injections?

Dr. Petty: No, we have seen no effects other than local irritation and formation of a lump in some people. We thought we had one "volunteer" who developed some kind of Henoch-Schönlein affair following the injection, but it proved to be unrelated, and he has received two injections since without difficulty. This, I am sure, is the same as the experience at Fort Detrick in immunizing more than 1,500 people.

ABSORPTION OF HIGH-MOLECULAR-WEIGHT MOLECULES BY THE LUNG—APPROACH AND RESULTS

Dr. Klaus Stemmer
University of Cincinnati

This research is concerned with the mechanisms that are involved in the absorption of molecules of high molecular weight by the lung, primarily in the lower respiratory tract and not the nasopharynx. The materials we are using are PVP, albumin, and botulinum toxin. We have worked mostly with aerosols containing albumin. This molecule has not only a high molecular weight but is generally of interest in medicine.

We have approached the complex problem from various viewpoints. None of the investigations are completed. I will outline the methods that have been used and present our findings from the electron-microscopic examination of the lungs of animals that had been exposed to an aerosol containing organic molecules of high molecular weight.

Methods.

Rats and dogs were used as experimental animals. They were exposed, by inhalation, in a hexagonal chamber. The chamber is in a room that is under negative pressure and equipped with its own exhaust system. The exposure is controlled from outside the negative chamber. This enables us to start and finish an experiment without entering the room housing the exposure chamber.

The control room also contains the equipment for the evoked-response technique. This technique was applied to investigate the earliest possible response of the animal to the toxic material. Since the molecules had molecular weights between 50,000 and 1,000,000, we thought that a response that would occur during the first few minutes of exposure could not be caused by the absorbed material but rather by contact between the material and the inner surface of the lung. We were first concerned if such a response would occur and, if so, what mechanism would be involved.

The techniques used are rather simple. The first employs chronically implanted electrodes in the visual cortex of the animal's brain. These electrodes were flashed every second by a photostimulator. The potentials were recorded from the implanted electrodes and stored in a computer. This computer eliminates the background changes and records only those potential

changes that are initiated by the light flash. These were added up in the pre-determined number and then plotted on an X recorder. The sum of 200 flashes was usually recorded. The response curves showed changes after a 1-hr exposure to an aerosol containing PVP or human albumin. Since both materials were considered essentially nontoxic, the aerosol might have caused subclinical effects on the eyes of the animals and, therefore, the change in the response curve. If we prevented contamination of the eyes of the animals by covering them with plastic, the results were the same. We concluded that the changes in the response curve had to be caused by contact between the materials and the inner surface of the respiratory tract.

The other technique being used is the electrophoresis of lung washings of animals exposed to the high-molecular-weight molecules. The electrophoretic fractions found in these washings are compared with the fractions found in the solution used for the aerosol and lung washings from control animals. The lungs are washed by aspirating 2 ml of normal saline into the lung. This is accomplished by removing the lung from the animal and suspending it by its trachea in a chamber. The pressure in the chamber can be changed from negative to positive, which causes first the aspiration of saline and then its expulsion. This is done three times. The electrophoresis is accomplished in gel columns. The instrument used is the disk-electrophoresis model by Canalco.

The experimental work has so far been conducted with an albumin aerosol only. The fractionation of the solution in the atomizer showed two distinct peaks in the albumin and postalbumin area. The fractionation of the control lung washings shows a peak in the prealbumin, the albumin, and the Beta complex. Further fractionation of these peaks has not been successful. The fractionation of the lung washings of the exposed animals showed a definite decrease in the Beta complex. There was also a definite peak in the post-albumin area. No prealbumin was present. These recordings have been qualitative up to this point. Work to quantitate these findings is in progress.

We do not have an explanation for the changes occurring in the fractions in the exposed animals. Our hypothesis is that the exposure stimulates the activity of either the tracheal and bronchial epithelium or the alveolar lining cells. This activity then causes the breakdown of the normally present Beta complex.

Electron Microscopy.

We examined the lungs of animals that had been exposed to an aerosol containing human albumin for 6 hr on 5 consecutive days. The tissue

was fixed in osmic acid and embedded in methacrylate. It was cut in the usual way. No stains were applied.

Figure 56 shows two indentations in one of the alveolar lining cells. These alterations have been described in the literature as pinocytosis. The outer membrane of the cell shows a fingerlike outgrowth. This feature will be seen in more detail in the following electron micrographs. We consider this as another type of pinocytosis.

Figure 57 shows several of the fingerlike structures marked by the single arrow. These structures are present at the surface of the alveolar lining cells and not in the capillaries. These outgrowing structures have been described at the junction between the endothelial cells. In our picture this might be demonstrated at the double arrow.

Figures 58 and 59 show a structure that might be described as an almost circular corpuscle. The nature of this is not known to us. It is present in both pictures.

Figure 58 shows it in the alveolar space in one area; in another area this structure is trapped by the fingerlike outgrowth seen in the previous pictures. Figure 59 shows a similar structure within the capillary.

Without knowing what this described circular structure actually is, it would be too early to consider the different "stages, " as represented in figures 58 and 59, as proof for the process of pinocytosis.

Assuming that the pinocytosis is the process by which large molecules are absorbed by the alveolar lining cells, we reasoned that this could be reflected in changes measured as cell potentials. To measure these electropotentials we constructed a double chamber. The two chambers were separated by a slice of lung tissue between 140μ and 160μ in thickness. The tissue was taken from lungs of goats, as close to the pleura as possible. We applied electrodes to the surface of the membrane on opposite sides in each chamber. The chambers were filled with Pinger's solution. We added albumin to one chamber after a baseline of the potential was established. We could find a certain rhythmic change in the magnitude of the measured potentials over a period of 45 min. We do not have any explanation for this change at this time. After each experiment, we determined the potassium, sodium, and chloride concentration in each of the chambers. There was no significant difference between their values before and after the experiment. The shift of the potassium, however, from the tissue into the solution on the side where the albumin was added was indicated but not statistically significant.

FIGURE 56

ELECTRON MICROGRAPH OF ALVEOLAR LINING CELL

Single arrow: indentation
Double arrow: fingerlike outgrowth

FIGURE 57

ELECTRON MICROGRAPH OF ALVEOLAR LINING CELL

Single arrow: fingerlike outgrowth
Double arrow: fingerlike outgrowth at junction
of endothelial cells

FIGURE 58

ELECTRON MICROGRAPH OF ALVEOLAR LINING CELL
SHOWING CIRCULAR CORPUSCLES (NATURE UNKNOWN)

Arrow at top center indicates corpuscle in the alveolar space.
Arrow at bottom right indicates this structure trapped by
fingerlike outgrowth.

FIGURE 59

ELECTRON MICROGRAPH OF ALVEOLAR LINING CELL SHOWING
CIRCULAR CORPUSCLE IN CAPILLARY

DISCUSSION

Dr. Wills (CRDL): I take it that you consider pinocytosis the probable principal mechanism of absorption.

Dr. Stemmer: That is correct. In this respect it was interesting to us to find in the literature some evidence that the accumulation of cations on the cell membrane could cause or stimulate pinocytosis. This shed some significance on our measurements in regard to changes in the potassium concentrations.

Dr. Sternberger (CRDL): Did you ever do any controls, slides, or micrographs for the normal cells that do not exhibit this sort of pinocytosis?

Dr. Stemmer: I would not say that it does not occur at all in normal cells, but it does not occur with this frequency, and the type of pinocytosis that I have seen in the normal lungs of control animals was the sort in which indentation or dips into the cell occurred, but not the outgrowths that I demonstrated.

Dr. Sternberger: There is some work in a recent issue of Cell Biology showing that surfaces of cells have structures similar to those you have described and that these structures appeared to be artifacts caused by osmium fixation. They have shown that there is a tremendous amount of dislocation of molecules as a result of osmium fixation.

Dr. Stemmer: I would argue this, since one can see the continuous membrane of these outgrowths. I do not think these are artifacts. You might have seen the paper published in the February issue of Histochemistry and Cytochemistry Journal in which pinocytosis of this type was described in cells in the intestinal tract and elsewhere.

Dr. Petty (Maryland Medical-Legal Foundation, Inc.): How did you determine potentials using implanted electrodes in a rat eye?

Dr. Stemmer: We did not implant them in the eye but in the visual cortex, and the potentials were recorded from the surface of the cortex.

Mr. Wilson (CRDL): I don't understand your membrane setup. Do I understand that you make a thin slice of lung and that you use it as a diffusion membrane?

Dr. Stemmer: For all practical purposes you might call it a diffusion membrane. We take the lung tissue close to the pleura, cut it at 160μ thickness, and place it between the two chambers. The electrodes are applied from the surface on both sides.

Mr. Wilson: I see. I imagined you would have half cells on either side rather than contact electrodes. Could the fluctuations pass for diffusion potentials due to concentration changes?

Dr. Stemmer: This is possible. One has to keep in mind that this is not a simple membrane but might consist of several alveoli packed together.

Mr. Wilson: It might also have holes through it.

Dr. Stemmer: We tested it for possible holes by adding dye on one side. If one bubbles oxygen on one side of the membrane, one can detect openings. With each gas bubble the membrane is moved and a little squirt of the dye goes through the hole to the other side.

CHOLINE-C^{14} AND ACh-C^{14} DISTRIBUTION AND ULTRASTRUCTURE OF BRAIN PARTICLES OBTAINED FROM MICE TREATED WITH TYPE A BOTULINUM TOXIN*
(Preliminary Report)

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Albany Medical College

The effects of Type A botulinum toxin have been associated with ACh release from nerve endings of various peripheral cholinergic nerves.**,† The effects of the toxin on the CNS have also been investigated recently,†† and evidences of change that could reflect an effect on synaptic transmission were recorded. The present study was undertaken to find biochemical and ultrastructural evidence for the involvement of a cholinergic system in the central effects of the toxin.

In addition, preliminary studies on the transport of toxin in different segments of the bowel suggested that maximum absorption of the toxin occurred through the stomach and upper small intestine. During acute studies, portions of the small intestine, liver, and skeletal muscle were also prepared for electron-microscopic examination.

All experiments were performed with female, Taconic, Swiss-Webster mice weighing between 18 and 20 gm. The experiments with Type A botulinum toxin were performed on groups of mice that had received 1 to 10 MLD50 units of toxin dissolved in phosphate buffer and injected ip in a volume of 0.1 ml/10 gm. Those animals showing signs of botulinum intoxication after 24 hr (belly drop, ataxia) and an equal number of control mice receiving only injections of phosphate buffer were used.

** Burgen, A. S. V., Dickens, F., and Zatman, L. J. J. Physiol. 109, 10 (1949).

† Ambache, N. Ibid. 108, 127 (1949).

†† Polley, E. H., Vick, J. A., Ciuchta, H. P., Fischetti, D. A., Macchitelli, F. J., and Montanarelli, N. Science 147, 1036 (   ).

All mice were sacrificed by a blow on the head. The brains were removed, pooled for each experiment, and placed in cold (5°C) sucrose solution (the composition is given below). The brains were homogenized for 2 min with a Teflon plunger revolving at 800 to 1,000 rpm. The clearance between this plunger and the smooth glass wall of the homogenizing vessel was 0.006 to 0.009 in.

Each homogenate was centrifuged at 1,000 g's for 10 min at 5°C. The supernatant from this centrifugation was divided into several 5-ml samples (one sample per mouse brain), and any remaining supernatant and the precipitate were discarded. The supernatant aliquots to be used in the experiments were preconditioned in a water bath (37°C) for 10 min and then incubated with choline-C^{14} (5×10^{-6}M) and acetyl-CoA (10^{-5}M) for 1 hr.

After incubation, the aliquots were centrifuged at 15,000 g's for 40 min in a precooled (5°C) Beckman Spinco L-2 ultracentrifuge using a No. 40 centrifuge head. The supernatant of this centrifugation was set aside and stored at 5°C while the precipitate was resuspended in 5 ml of fresh sucrose solution and recentrifuged at 15,000 g's for 40 min. The wash supernatant was combined with the original supernatant and centrifuged at 100,000 g's for 40 min. The final supernatant was discarded and the 15,000- and 100,000-g precipitates were taken for analysis.

Extraction and Separation of Choline-C^{14} and ACh-C^{14}.

One milligram each of carrier choline chloride and carrier ACh chloride, both dissolved in 1 ml of distilled water, were added to each sample. The precipitates were resuspended and the contents of each tube adjusted to pH 2.5 to 3.0 with 1 drop of 2 M HCl, placed in a water bath for 1/2 hr at 37°C, and, afterward, centrifuged at 10,000 g's for 15 min at room temperature. The supernatant of this centrifugation was set aside while the solid white matter was washed with 1 ml of distilled water. The combined supernatant and wash were spread out on a 7 X 9 in. sheet of Whatman No. 4 chromatography paper and washed by chromatographing with diethyl ether. All chromatography was done in sodium-free borosilicate tanks. The material on the paper was then accumulated at one end of the paper by chromatographing with 0.001 M HCl. The bank of accumulated material was distributed by ascending chromatography with a solvent consisting of butanol:acetic acid:water in a ratio of 7:2:2, v/v. This divided the material into choline and ACh bands that were eluted and counted for 10,000 counts with a thin-window, gas-flow Geiger counter equipped with an anticoincidence background shield (Nuclear-Chicago C-115 system;

efficiency of 10. 3% for samples prepared as stated). The advantage offered by this system is that the fate of both choline and ACh can be studied simultaneously. The specific activity of radio-labeled compounds, however, could not be determined since endogenous carrier choline was undoubtedly present. All results were, therefore, expressed as counts per minute.

Chemicals and Solutions.

Methyl choline-C^{14} chloride (37.6 mc/mM) was obtained from the Nuclear-Chicago Corp. and was diluted upon receipt with anhydrous methanol and stored at $-18^{\circ}C$. Carrier ACh chloride and choline chloride were obtained from Eastman Chemical Co. and physostigmine sulfate was obtained from Merck and Co.

The sucrose solution was made up of the following reagent-grade chemicals: sucrose, 102.6 gm/l; 1-cysteine·HCl, 6 gm/l; physostigmine sulfate, 30 gm/l, dissolved in 0.05 M potassium phosphate buffer (pH 7.0).

Electron Microscopy.

Electron micrographs were prepared from both the 15,000- and 100,000-g precipitates of the brain homogenates of both control and toxin-treated mice. The samples were fixed in glutaraldehyde, processed, and embedded in epon. The sections were stained with lead citrate and photographed with an RCA E.M.E. 3G electron microscope.

At sacrifice, portions of the small intestine and liver were immediately fixed in glutaraldehyde. Thereafter, the specimens were processed, embedded in epon, cut, stained, and photographed.

Results and Discussion.

The effects of the toxin were always compared to the subcellular distribution of choline-C^{14} and ACh-C^{14} in control animals drawn from the same animal pool on the same day. The homogenates of all animal brains appeared to utilize choline-C^{14} and acetyl-CoA for formation of ACh-C^{14}. Both the 15,000- and 100,000-g fractions obtained from normal and toxin-treated animals contained choline-C^{14} and ACh-C^{14}, even after repeated washings. Therefore, the particulate matter of each subfraction could both sequester and concentrate these substances, since the concentration of the radioactivity in the particulate matter was from 5 to 25 times greater than that of the supernatant fluid.

Table 38 shows that the radioactivity of the 15,000-g fraction of the toxin-treated animals was almost always less than that of the control mice. The 100,000-g fraction always showed the opposite relationship. In this fraction, the choline-C^{14} and ACh-C^{14} were always greater than the control mice. All differences were statistically significant at $P < 0.05$.

TABLE 38

CHOLINE-C^{14} AND ACh-C^{14} LEVELS IN PARTICULATE FRACTIONS OF MOUSE-BRAIN HOMOGENATES

No. of mice	Treatment	15,000 g's		100,000 g's	
		Choline	ACh	Choline	ACh
		counts/min			
4	Control	3,441	983	—	—
4	Toxin	3,244	724	—	—
4	Control	3,071	1,205	—	—
4	Toxin	2,668	895	—	—
4	Control	4,272	403	1,755	93
4	Toxin	3,846	248	2,014	124
4	Control	2,803	1,260	1,997	965
4	Toxin	1,604	615	2,778	1,313
2	Control	3,395	476	2,778	240
2	Toxin	2,647	392	3,156	309
2	Control	4,708	501	2,776	431
2	Toxin	4,058	452	2,932	549
2	Control	5,071	669	3,596	580
2	Toxin	4,187	649	4,060	600
2	Control	5,263	578	4,152	538
2	Toxin	3,884	601	4,239	667

The photomicrogra... is of both the control and treated 15,000-g fractions showed pinched-off ...rve endings, mitochondria, endoplasmic reticulum, and a certain amo...it of cellular debris. The nerve-ending portion in the photomicrographs from toxin-treated mice appeared to contain fewer vesicles, and, frequently, those present were markedly distended.

The photomicrographs of the control and treated 100,000-g fraction contained portions of endoplasmic reticulum, numbers of small vesicles, clusters of ribosomes, and debris. They were free of pinched-off nerve endings, indicating good separation of free vesicles and vesicles trapped within nerve-ending particles. There were no apparent differences between the photomicrographs of the 100,000-g fractions of the control and toxin-treated mice.

It is difficult to quantitate the apparent reduction in the number of vesicles within the nerve endings of the treated mice because of variability in numbers and distribution in each ultraphotograph. The general impression gained by studying multiple photomicrographs from many blocks, however, is firm. Furthermore, the method of isolation does lead to structural artifacts, evidenced by mitochondrial swelling and moderate distortion of the cristae. This apparent reduction in number of vesicles may represent either a direct quantitative reduction or indirect reduction because of increased fragility of these vesicles following the administration of toxin.

Sections of the synaptic junctions in normal skeletal muscle have been studied. Only preliminary observations on this structure after toxin treatment of the host are currently available. Zacks¤ and coworkers,* however, failed to demonstrate any change ultrastructurally after host exposure to toxin.

Sections of the small intestine show a number of vesicles and swelling of the endoplasmic reticulum immediately above and below the nucleus in the living epithelium. In some sections, there is the suggestion of replication of endoplasmic reticulum. The microvilli and the remainder of the internal organelles are intact. The basement membrane, small capillaries, and supporter cells beneath the epithelium are not remarkable. On review, this study would suggest that a general shift in the cholineacetylase enzyme and the choline and ACh sequestering particles from the 15,000- to the 100,000-g fraction took place in the toxin-treated mice without any difference in the total enzyme. Electron-microscopic study of these fractions supports these results

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* Zacks, S. A., Metzger, J., Smith, C. W., and Blumberg, J.M. J. Neuropathol. Exptl. Neurol. 21, 610 (1962).

by demonstrating an apparent reduction in the number of vesicles in the nerve endings. Because of the technical difficulties in evaluating the particle preparations with the electron microscope, additional experiments, designed to study the synaptic junctions in situ by specific brain biopsies in control and toxin-treated mice, are in progress.

DISCUSSION

Dr. Wills (CRDL): We come now to one of the most vexing questions in relation to botulinum: whether botulinum toxin does have a specific effect on central nerve cells. We heard earlier that iv injection of the toxin does nothing for several hours. Later we will hear from another group of workers in the same laboratory that iv injection of botulinum toxin produces very apparent immediate effects. Other than this last report, I do not know of any very clear demonstration of botulinum toxin on nerve cells except for a paper by Potts that came out in 1959, in which he showed that there were some effects of botulinum toxin on the CO_2 production and the respiratory quotient of cells of the cerebrum and the medulla. These effects are both increases and these tend to be in the opposite direction from the kind of action that might be supposed to occur on the basis of the work that will be reported now. These guesses about metabolic activity, however, are not always very good. We find not infrequently that gross activity is not correlated with metabolic activity. Therefore, the Potts report is not necessarily a contraindication of the conclusions to be presented now by Capt. Vick.

CENTRAL AND PERIPHERAL EFFECTS OF TYPE A BOTULINUM TOXIN

Capt James A. Vick (with Capt Henry Ciuchta and Sp5c James H. Manthei)
Experimental Medicine Division
CRDL

I would like to report on what we think may be a CNS effect of botulinum toxin, Type A. Recent studies have indicated that botulinum toxin may have some CNS effects. Animal studies by Michaelov and human poison cases described by Dr. Tyler have both suggested that brain-stem or spinal-cord effects may be the result of poisoning by botulinum toxin. Several clinical reports, including those by Dolman, Dack, and Rogers, have all indicated that the problem of differential diagnosis of botulinum intoxication implies some dysfunction of the CNS. This, of course, is of primary interest to us. I was also pleased to be able to ask Dr. Stemmer more about his report of visual-area recordings in the rat after administration of the toxin. The effect observed in their preliminary studies is much like that seen after the administration of an anesthetic; in other words, a marked depression of these areas of the brain.

Table 39 shows data for 26 monkeys, 12 anesthetized with pentothal sodium and 14 unanesthetized. The anesthetized monkeys received from 5 to 50 LD50's of botulinum toxin. The time to death is also indicated. In the unanesthetized animals, the electrodes were implanted for electroencephalogram (EEG) recording approximately 21 days before the study. At the bottom is the definition of LD50 as used in this study, 50 MU/kg of body weight. The initial concentration of the toxin, determined by mouse bioassay, was 30,000 MU/ml, suspended in phosphate-buffered gelatin. This was diluted for use in normal saline to a final concentration of 100 MU/ml and injected iv.

Figure 60 shows a very early picture of what was observed after administration of 5 LD50's of botulinum toxin to the anesthetized monkey. We recorded a surface EEG on the right and left sides, an integrated heart rate, respiration, blood pressure, and the ECG. The ECG shows very little except that there were no gross abnormalities. This is a slow tracing taken over 9 min postinjection. At first there is very little change in the basic physiology of this monkey. There is a slight transient fall in blood pressure, and an abrupt, but not significant, change in respiration. A progressive loss of cortical electrical activity, however, occurs. This went on and, at approximately 10 to 15 min, showed a completely isoelectric tracing. This was often followed by recovery and a sequence of events that will be shown in figures 61 through 74.

TABLE 39

DATA ON ANESTHETIZED AND UNANESTHETIZED MONKEY PREPARATIONS TREATED WITH 1 TO 50 LD50's OF BOTULINUM TOXIN

Anesthetized preparations				Unanesthetized preparations			
No.	Date	Dose	Survival time	No.	Date	Dose	Survival time
		LD50	hr			LD50	hr
1	23 Oct	50	12	1	16 Jan	20	20
2	29 Oct	50	7	2	23 Jan	10	19
3	12 Nov	50	10	3	30 Jan	4	28
4	15 Nov	50	24	4	6 Feb	3	31
5	19 Nov	50	22	5	18 Feb	5	18
6	20 Nov	50	10	6	9 Mar	1	40
7	21 Nov	50	12	7	1 Apr	50	8
8	27 Nov	50	30	8	28 Apr	5	10
9	3 Dec	50	11	9	29 Apr	5	18
10	8 Dec	50	13	10	13 May	5	21
11	11 Dec	5	31	11	20 May	5	11
12	15 Dec	5	30	12	3 Jun	1	38
				13	22 Jul	5	8
				14	14 Aug	25	Permanent

Note: One monkey iv LD50 = 50 mouse ip LD50's (MU). Initial concentration of toxin, as determined by mouse bioassay, was 30,000 MU/ml of phosphate-buffered gelatin. This was diluted in normal saline to final concentration of 10 MU/ml for iv injection.

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EEG, right side

EEG, left side

Heart rate

Respiration

Blood pressure

EKG
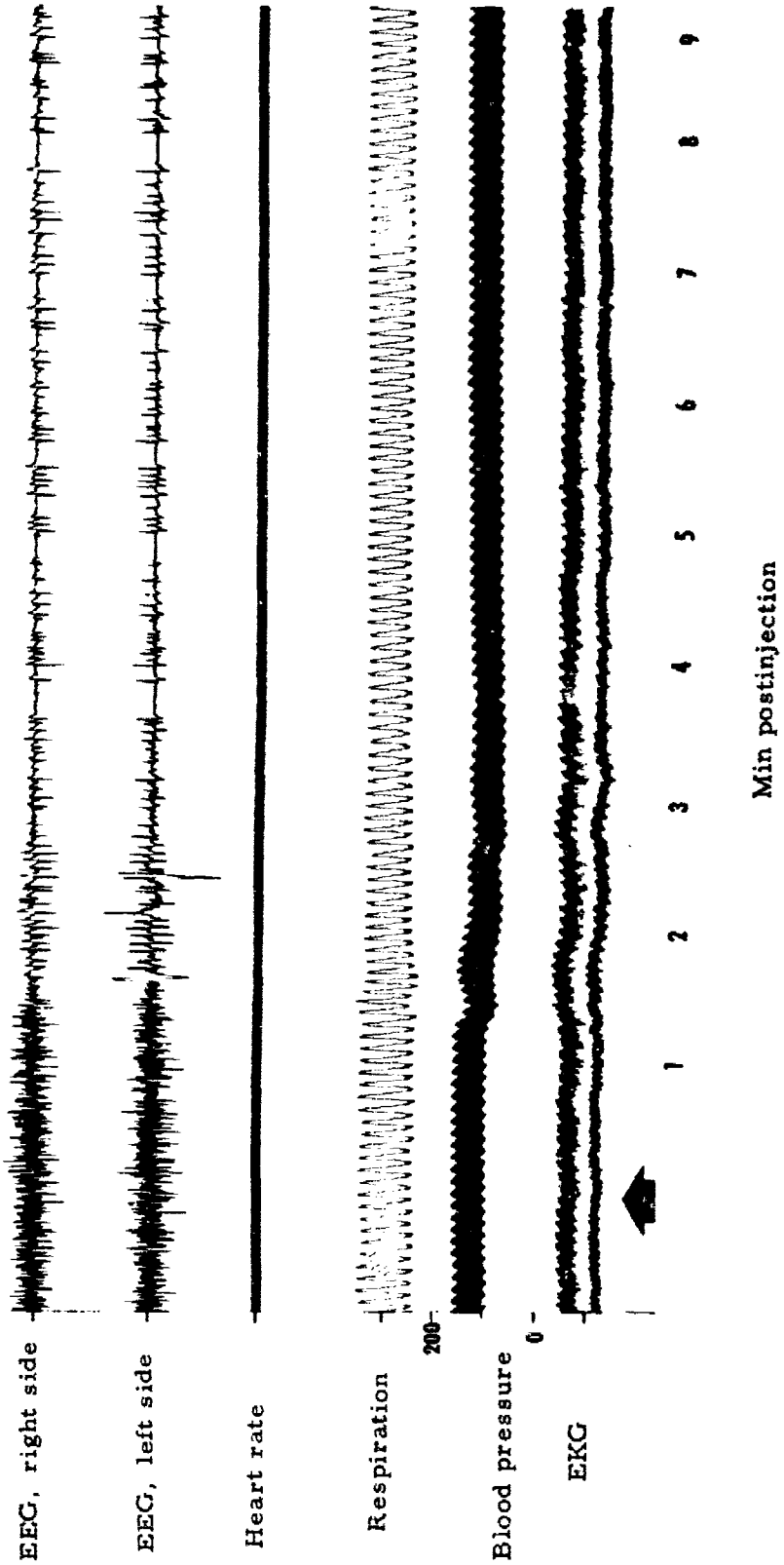
Min postinjection

FIGURE 60

PHYSIOLOGIC DATA AFTER ADMINISTRATION OF 5 LD50'S OF BOTULINUM
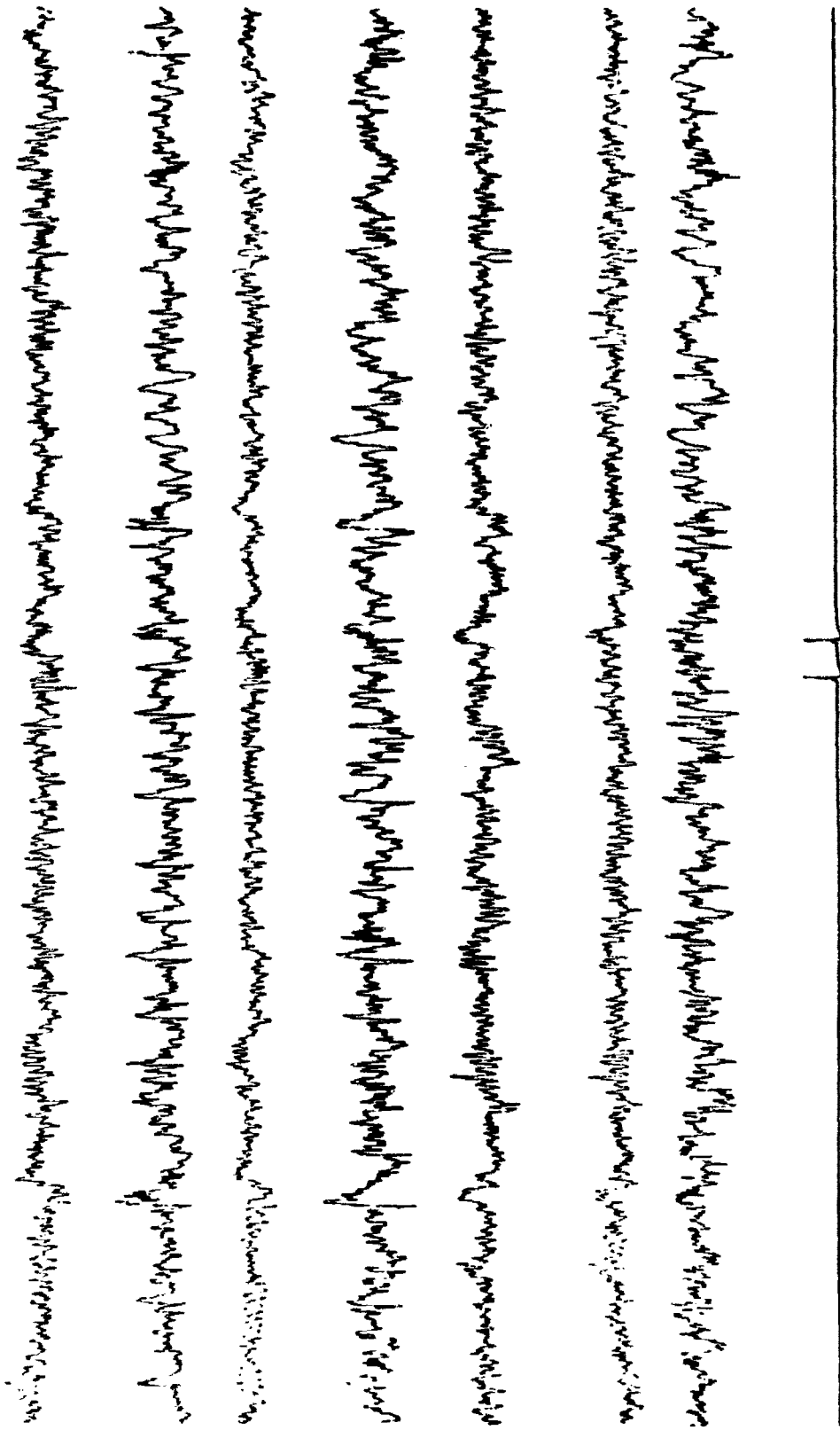TOXIN TO ANESTHETIZED MONKEY

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FIGURE 61

EEG CONTROL TRACING FROM LIGHTLY ANESTHETIZED MONKEY "MORRIS"
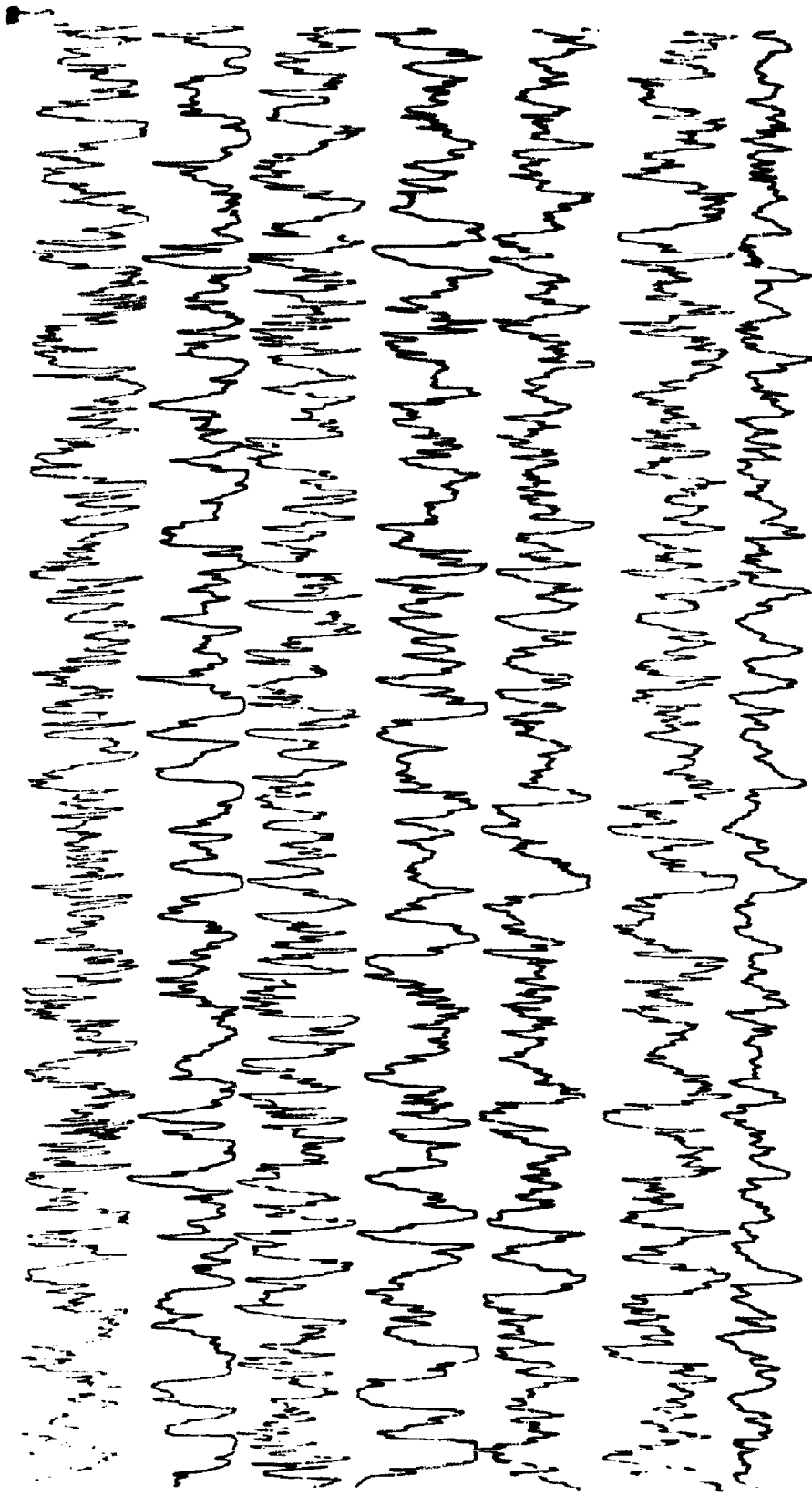
(Electrode placement is shown)

FIGURE 62

EEG 1 MIN POSTINJECTION OF 50 LD50'S OF BOTULINUM TOXIN
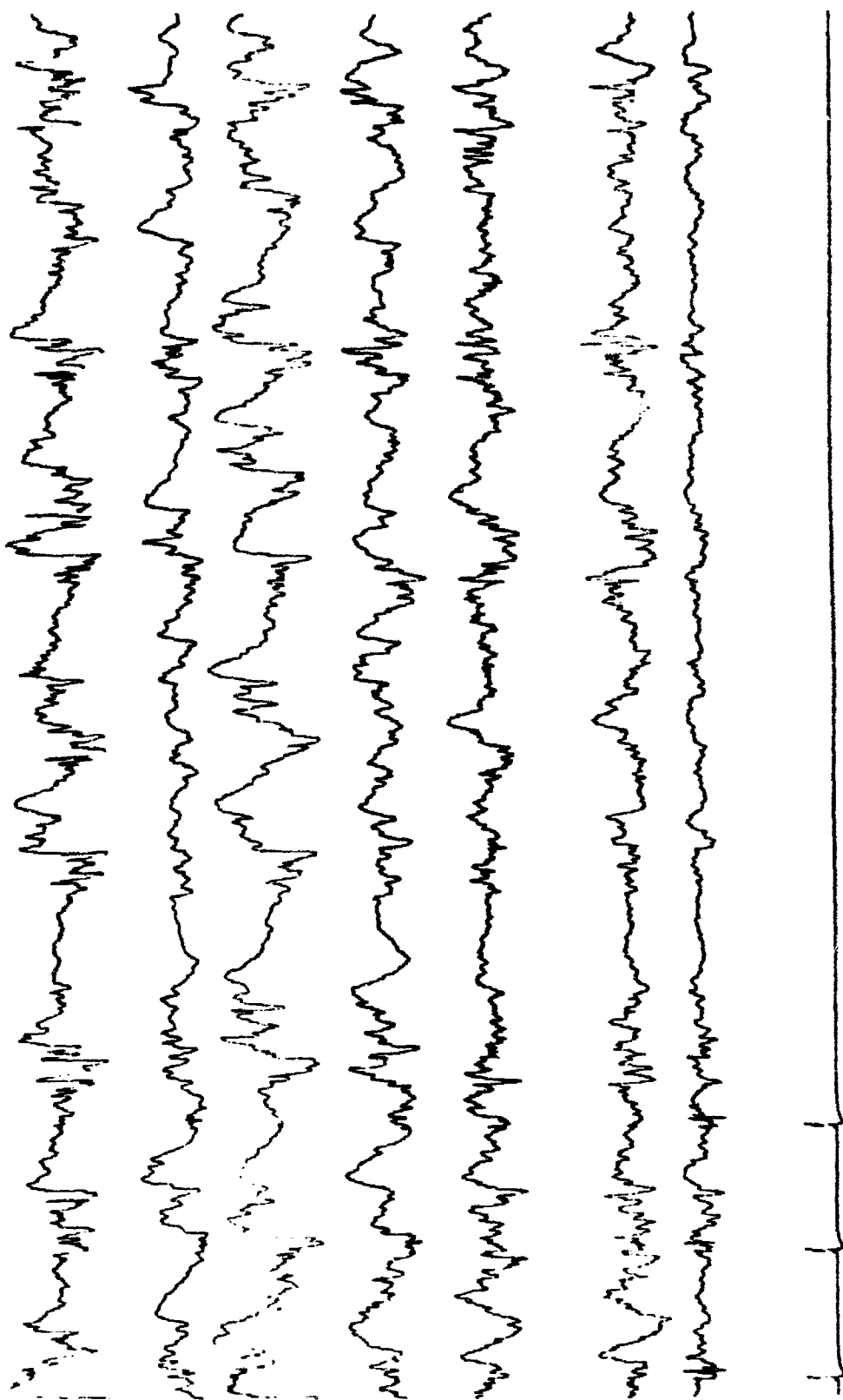
(Marked changes are evident)

FIGURE 63

EEG 5 MIN POSTINJECTION

(Further decrease in cortical activity, many slow waves and little high-
frequency activity; effects of intermittent photic stimulus are evident)

266
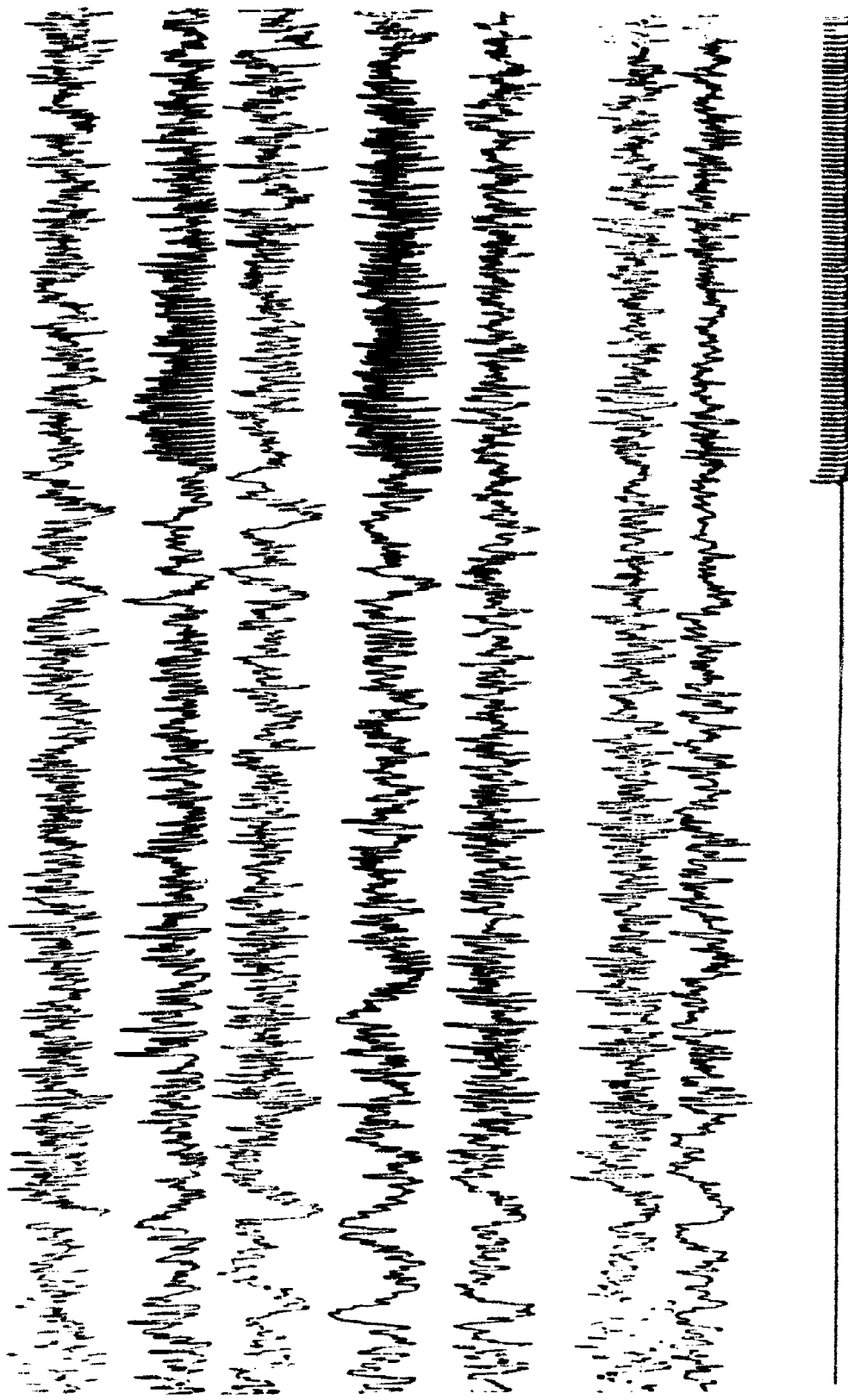
FIGURE 64

EEG 2 HR POSTINJECTION

(Some cortical activity has reappeared and, as often happens from 1 to 7 hr after poisoning, these fluctuations seem to be linked to depth of anesthesia)
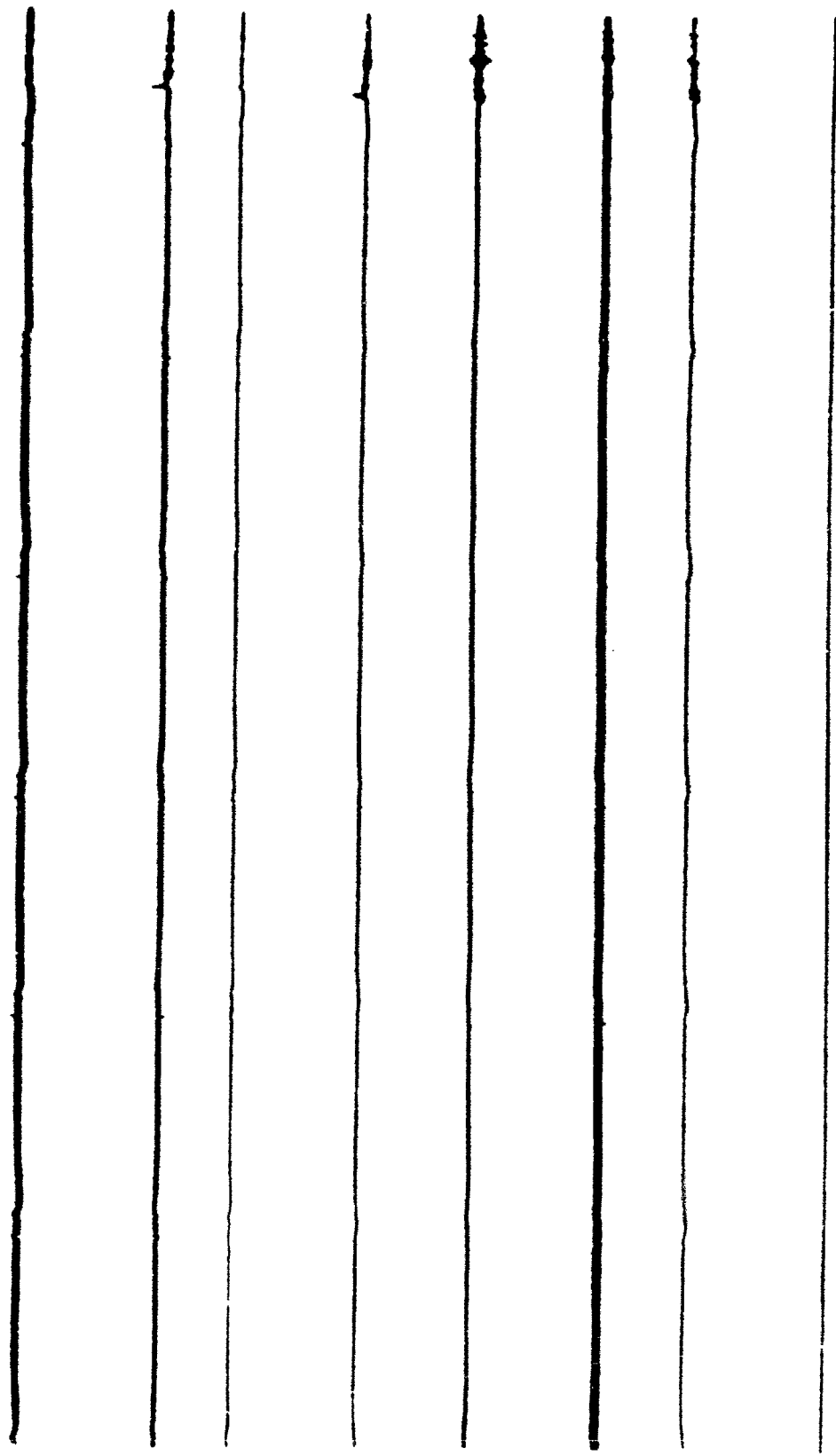
FIGURE 65

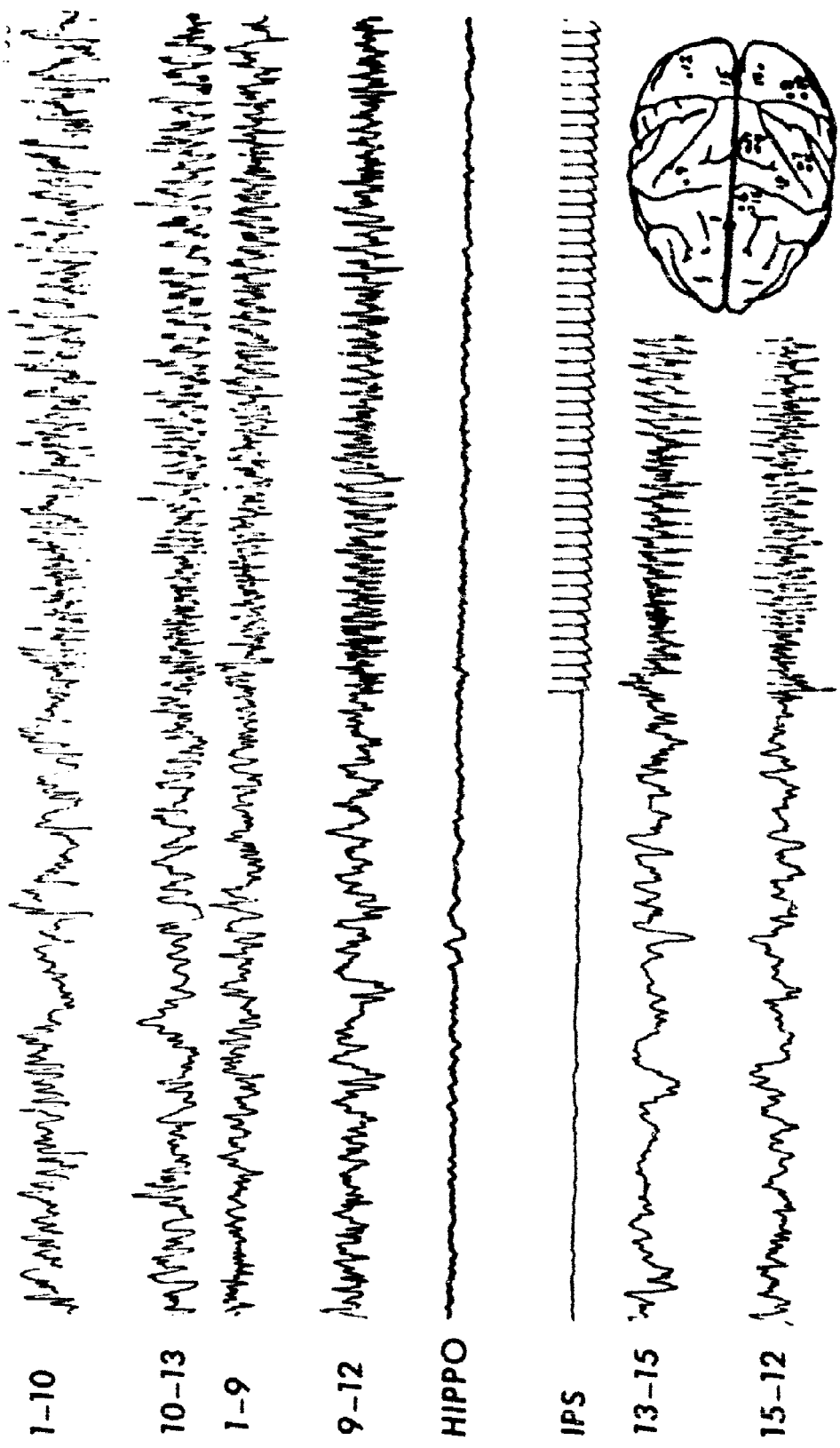**EEG 5-1/2 HR POSTINJECTION AND 30 MIN BEFORE DEATH**

(Cortical activity lost)

1-10

10-13

1-9

9-12

HIPPO

IPS

13-15

15-12

FIGURE 66

EEG CONTROL TRACING FROM UNANESTHETIZED MONKEY "GRANNY," WITH
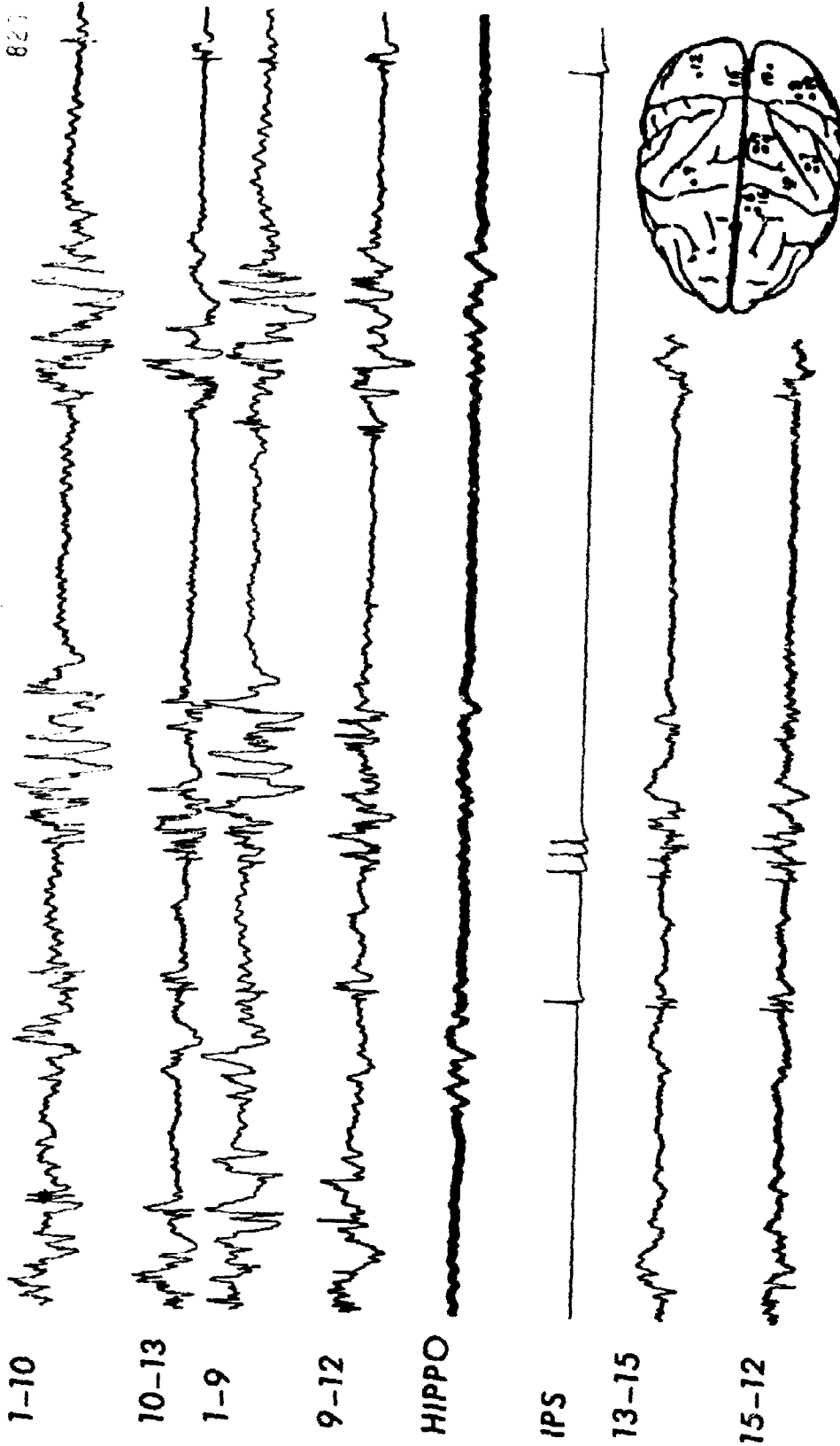EFFECT OF PHOTIC STIMULUS APPEARING MIDWAY THROUGH TRACING

FIGURE 67

EEG 50 SEC POSTINJECTION OF 50 LD50'S OF BOTULINUM TOXIN

(The very rapid changes may be indications that toxin, or its products, penetrate "blood-brain" barrier)
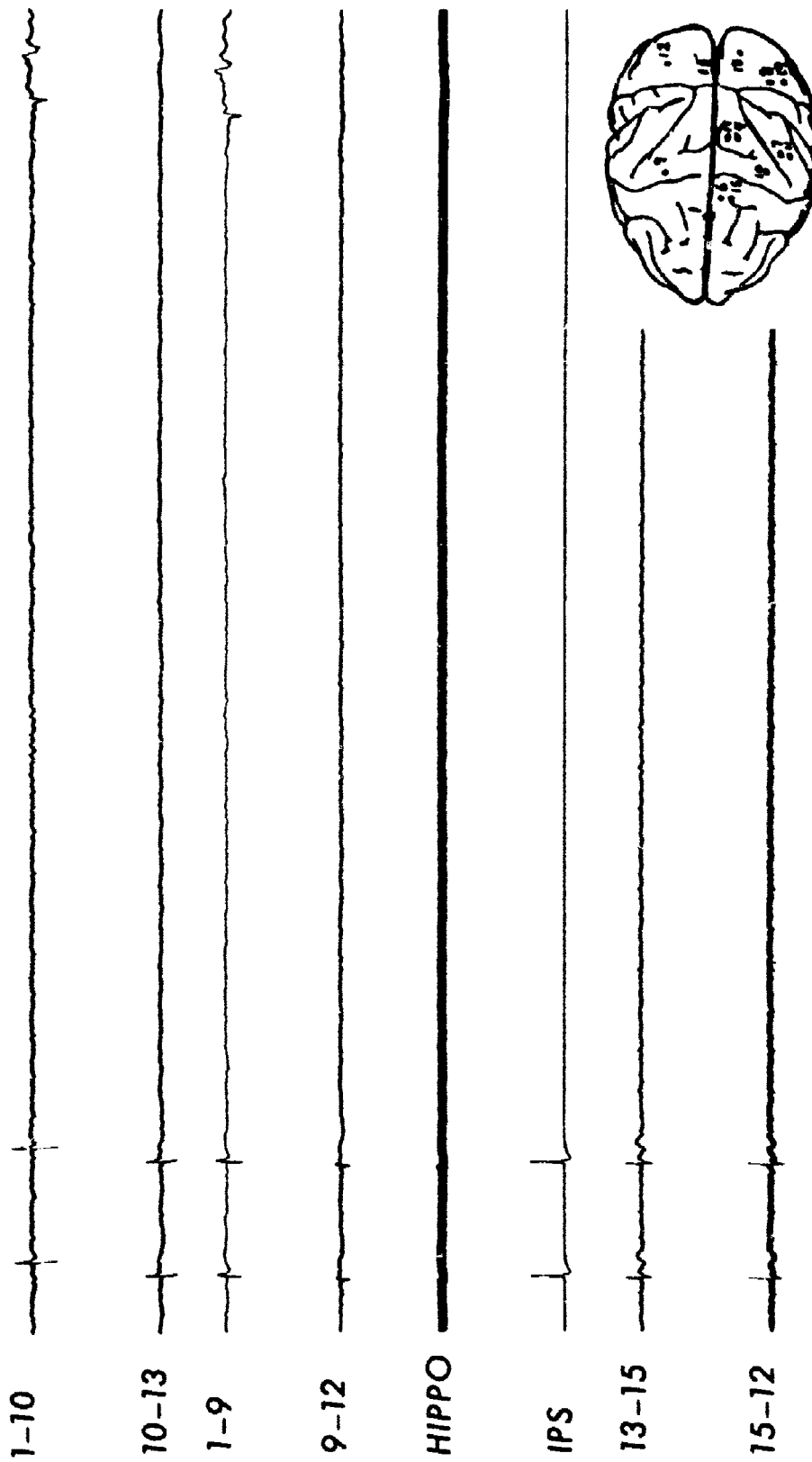
1-10

10-13

1-9

9-12

HIPPO

IPS

13-15

15-12

270

1-10

10-13

1-9

9-12

HIPPO

IPS

13-15

15-12

FIGURE 58

EEG SHOWING COMPLETELY ISOELECTRIC STATE 5 MIN POSTINJECTION
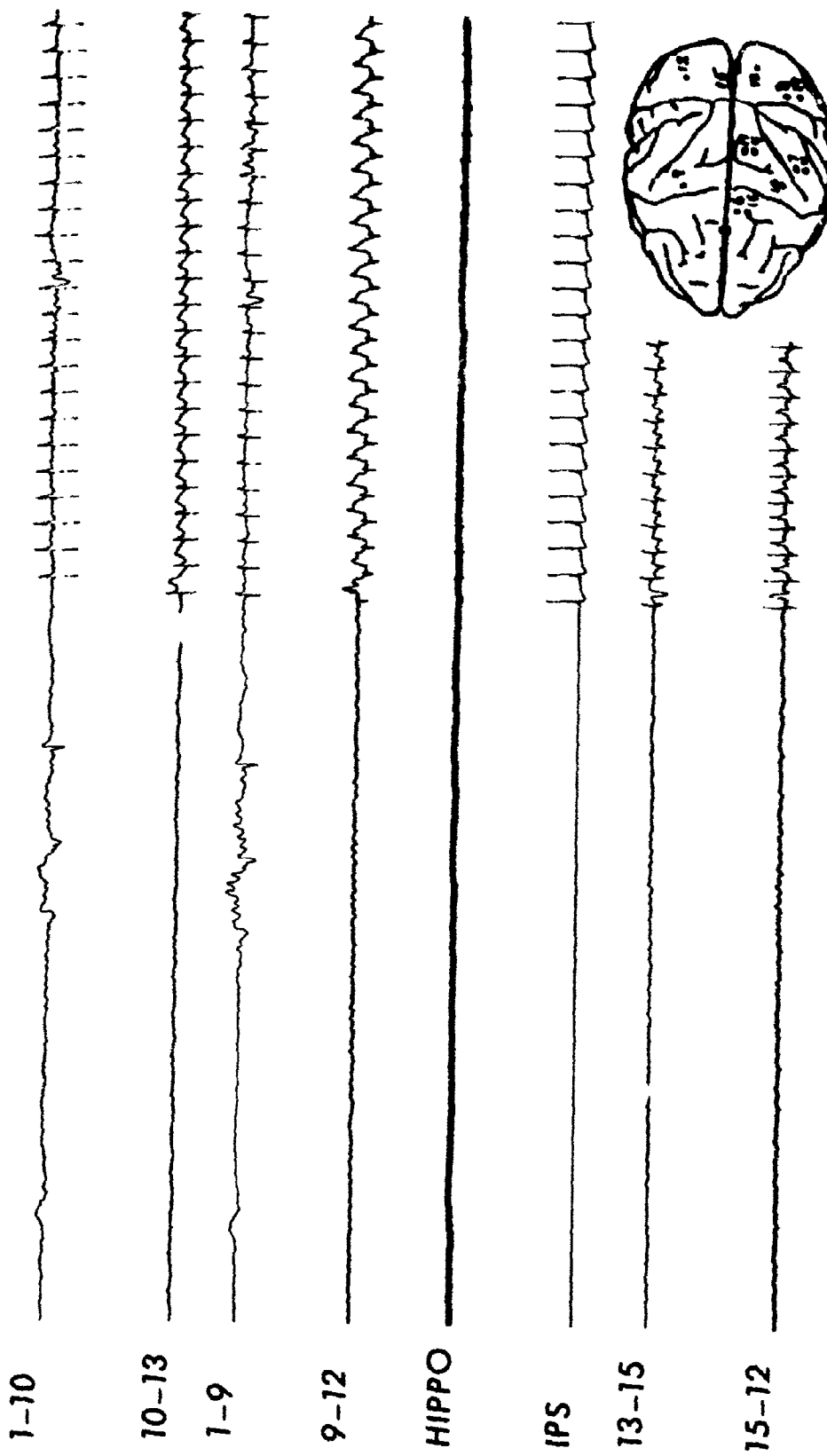
271

1-10

10-13

1-9

9-12

HIPPO

IPS

13-15

15-12

FIGURE 69

EEG SHOWING PARTIAL RECOVERY AND RESPONSE OF
MONKEY TO PHOTIC STIMULI AT 15 MIN
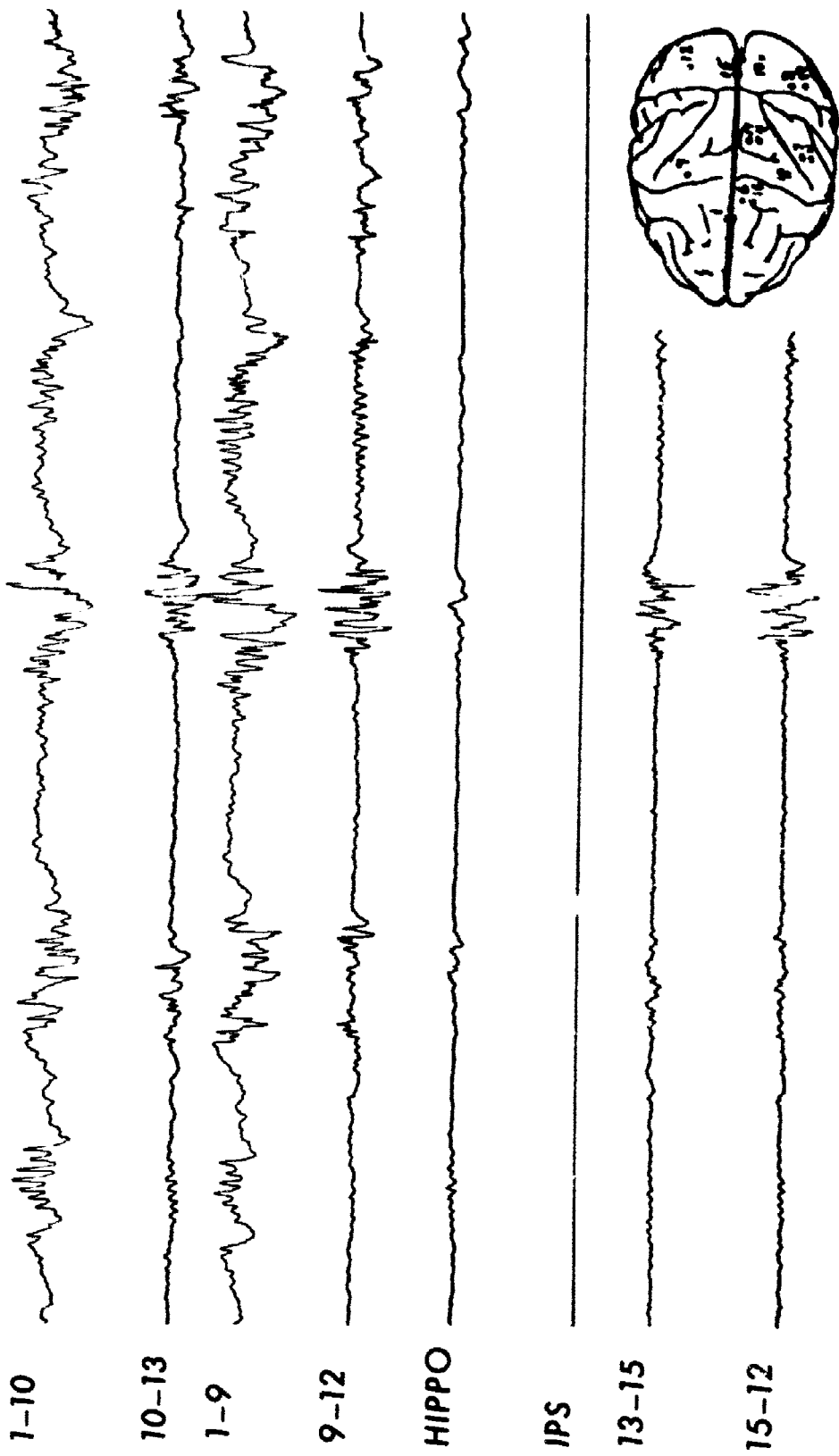
FIGURE 70

EEG SHOWING FURTHER RECOVERY AT 65 MIN

1-10
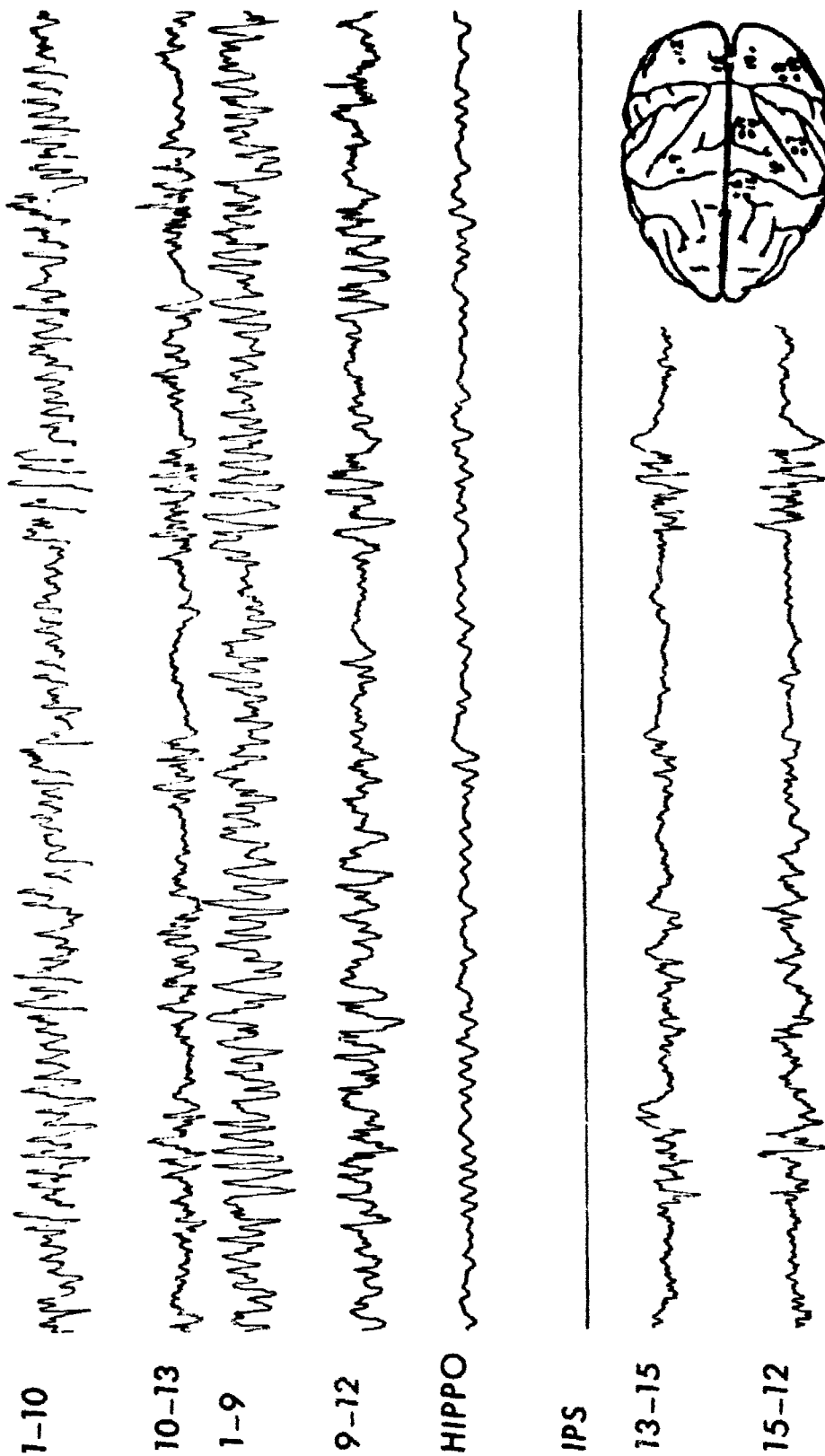
10-13

1-9

9-12

HIPPO

IPS

13-15

15-12

FIGURE 71

ALMOST NORMAL EEG AT 90 MIN

1-10

10-13

1-9

9-12

HIPPO

IPS

13-15

15-12
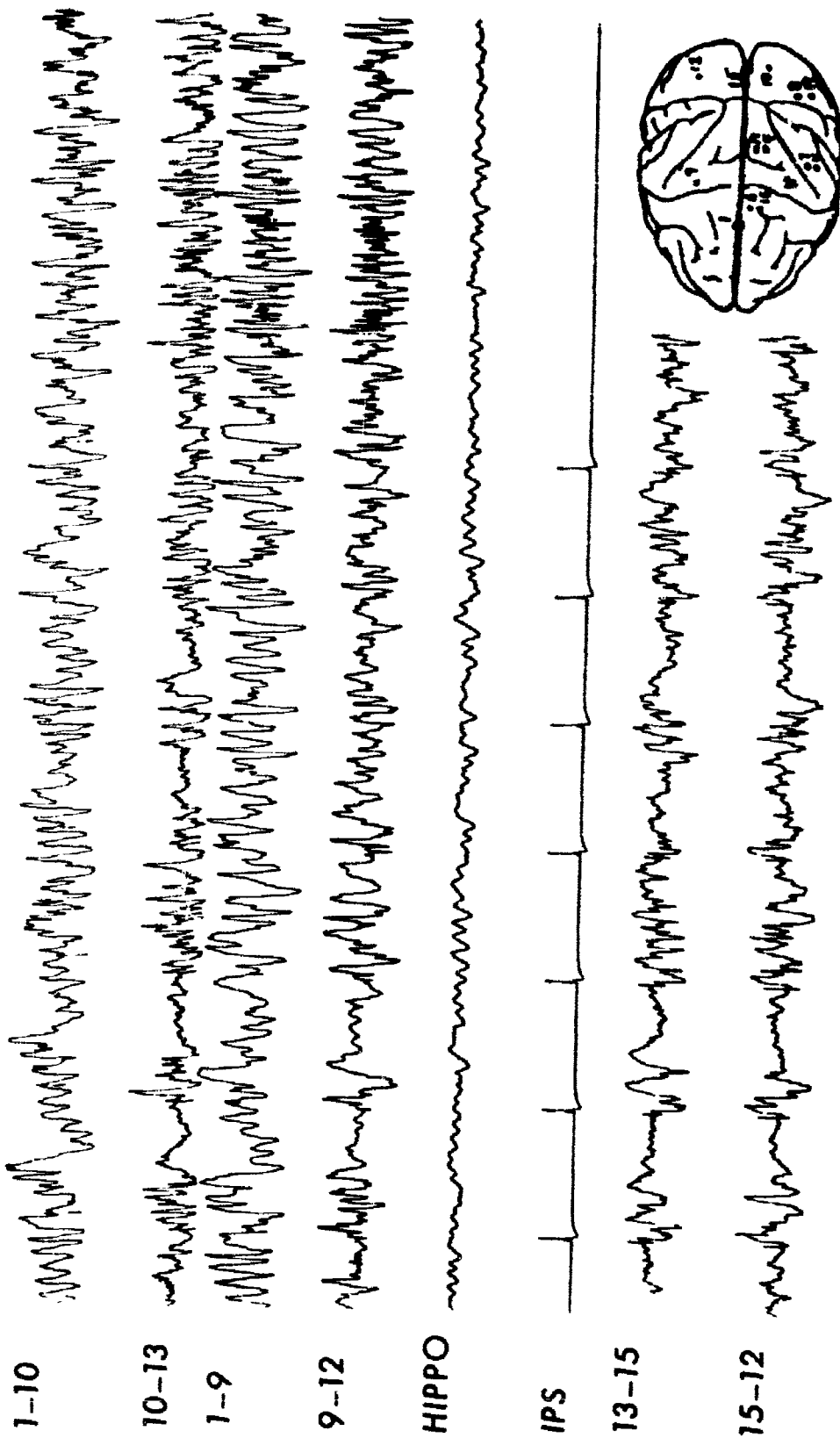
FIGURE 72

NORMAL EEG AT 5 HR, AT WHICH TIME ANIMAL WAS
ABLE TO EAT AND DRINK

1-10

10-13

1-9

9-12

HIPPO

IPS

13-15

15-12

275

FIGURE 73

EEG SHOWING SPONTANEOUS LOSS OF MUCH CORTICAL ACTIVITY AT 10 HP.

(There is no explanation for this at present)

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1-10

10-13

1-9

9-12

HIPPO

IPS

13-15

15-12

FIGURE 74

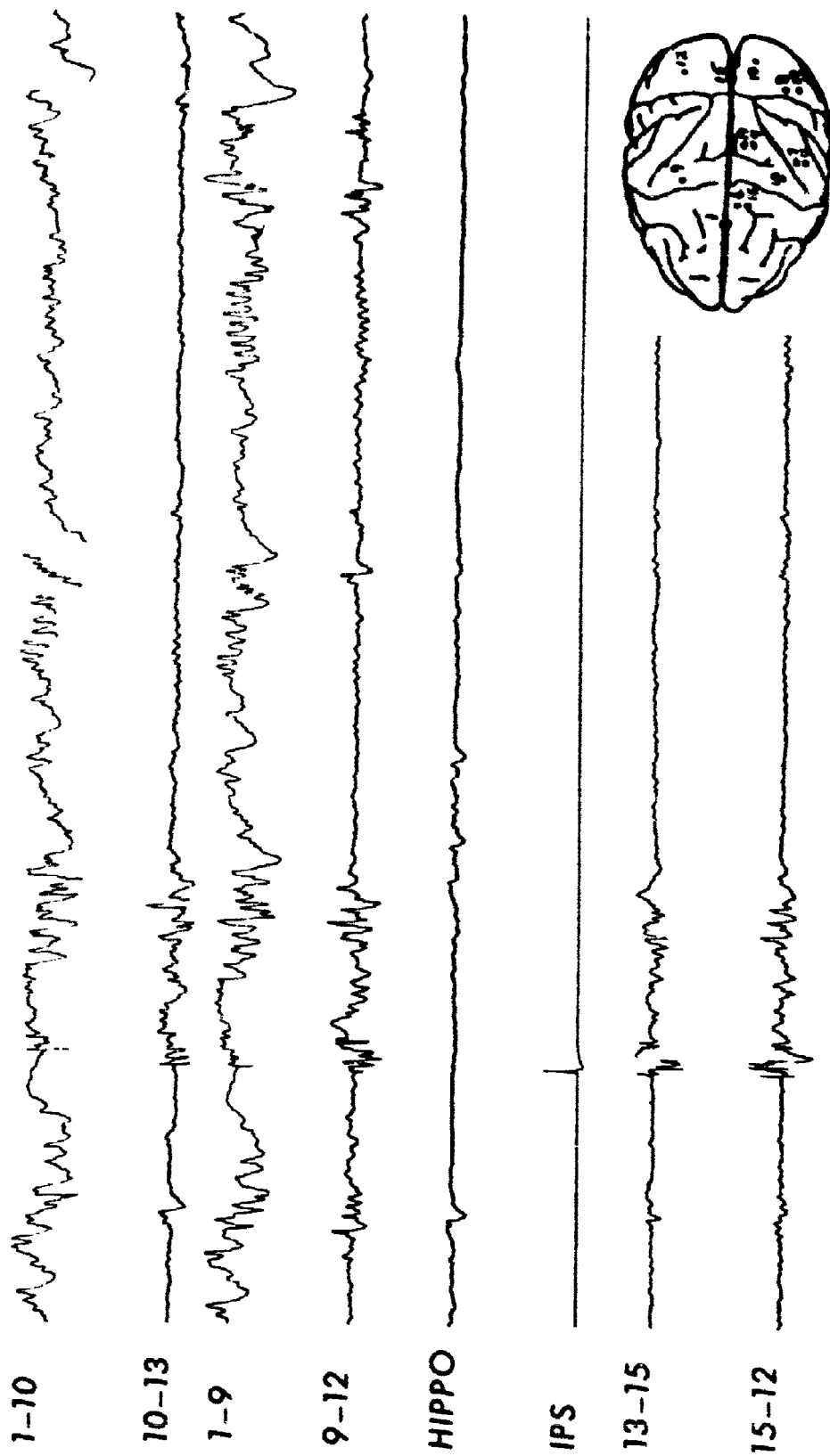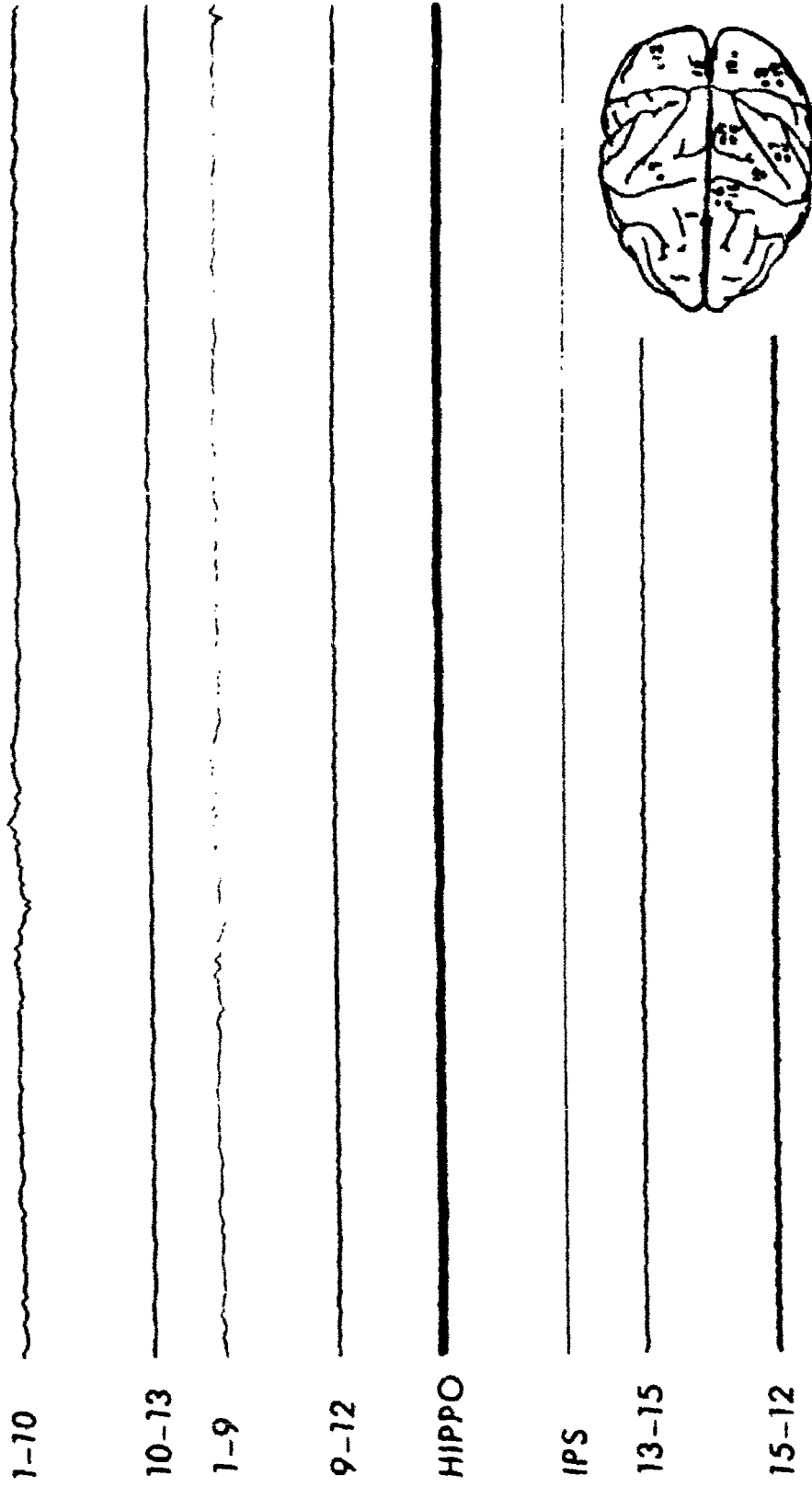EEG AT 18 HR AND JUST BEFORE DEATH FROM RESPIRATORY FAILURE

Concerning the recording of the various parameters, I have a very short film titled Polygraphic and Electroencephalographic Study of Chemical Agents that will show how we prepared the animals.

The loss and recovery of cortical electrical activity occur more strikingly in some animals than in others, usually associated with partial or complete unconsciousness. We observed and have characterized the usual respiratory mechanism of death. In some monkeys, we recorded the phrenic-nerve discharges originating in the respiratory center. The distal end of the cut nerve was stimulated to ascertain whether the neuromuscular junction was intact. We found that the respiratory center continued to function even as the block developed at the diaphragm. Artificial respiration at the time of respiratory paralysis, however, did prolong survival. All animals eventually died of cardiovascular collapse.

DISCUSSION

Dr. Tyler (Peter Bent Brigham Hospital): Can any substance, chemical or otherwise, change the EEG acutely? I would say yes, oxygen, because if you deprive an animal of oxygen completely, in 12 sec you get flattened EEG. There are chemicals that can do it, and I sometimes think it fallacious to talk about blood-brain barriers. There is a blood-brain barrier for every chemical, there is no one blood-brain barrier, and no one has defined the blood-brain barrier for botulinum. Therefore, we cannot talk about a blood-brain barrier as such, only for a given substance, what resistance does it have, and so forth.

The second problem is to define whether the toxin or whether "something" in the preparation is active because we heard that huge total doses of toxin given iv produced no clinical symptoms in another experiment. Certainly, the EEG's presented this afternoon are quite striking. One wonders if the methods of preparation of the two toxins were identical.

We have given iv toxin to dogs, cats, rats, and rabbits and could not reproduce any acute effect as reported this afternoon. We have been using, however, the crystalline toxin in acetate. Dr. Vick was using the "mud" or phosphate-buffered-gelatin toxin. The whole question is what is in the syringe and why two similar toxin preparations should give opposite results. All of us are going to have to be extremely careful to reproduce exactly the situation and techniques of each other. The theory behind the central effect of botulinum is very suggestive.

Dr. Sternberger (CRDL): I am not sure it is true that Dr. Streett used the same batch of toxin as Dr. Vick used. Did you use the same batch? At least this variable could be taken care of by having used the very same batch.

Capt Stookey (CRDL): We just finished testing a group of nine monkeys, trying to shoot for 100 LD50's in them, which we practically reached. We used the preparation from Mr. Vocci's section, we did not dilute it or do anything to it. A bioassay in mice of an aliquot of the same sample showed that we gave our monkeys 100 LD50's. We gave it iv in the same solution that you saw in the movie this morning. The monkeys were restrained in a sitting position, and believe me they never slowed down. They kept right on going. In addition, Capt Vick reported that some of his monkeys died in a period of 7 hr. None of ours showed any symptoms until at least after 7 hr, and some of these died at 15 hr, others lived over 30 hr. Those that died at 15 hr, however, did not show any symptoms at 7 hr. They went over the hill extremely rapidly. The Vapor Toxicity Branch, I understand, has given 75 monkeys iv injections and has never observed this anesthetic or depressant effect.

Dr. Wills (CRDL): This still doesn't answer Dr. Sternberger's question, which was: Have you used the same preparation of toxin that Capt Vick used?

Capt Stookey: I understand that his was in a different vehicle.

Dr. Streett (CRDL): I think Mr. Vocci could best answer that because we can't tell if it is the same batch or not; we just request it. One other variable that is in it is that they are diluting with saline, and the only thing that ours ever had has been the gelatin buffer the way we received it. I also notice that you said you dilute down to 10 MU/ml. In the film, the one that I think got 50 LD's, what volume did it get?

Capt Vick: For purposes of photography, that animal got saline at the supposed time of injection. When you are filming, all things are not optimum; therefore, the injection of botulinum was actually made after we had filmed the injection of saline for photographic purposes.

Dr. Streett: Yes, I mean in that one.

Capt. Vick: What, the volume?

Dr. Streett: Yes, receiving 50 LD50's, what volume did it receive this in?

Capt Vick: In the preparation that received 50 LD50's, the dilution was not made to that concentration. The dilution varied with the amount we were going to give so that we could maintain a constant volume of injection. I would have to calculate back to give you the exact amount.

Also, it seems to me that we have a controversy; I recall not too many months ago, Dr. Streett, that using the substance you and I and others had been testing, you injected one of your monkeys in the femoral vein. Would you describe briefly what you saw that day?

Dr. Streett: We saw a monkey that slowed down considerably but did not go to sleep. I cannot vouch, however, for what was in the syringe. We have found, I agree, that you are finding something, and I think it is actually significant, but that monkey did not go completely to sleep. He sat in the corner of the cage and did not move around as much as the others. Everyone also saw our monkeys on the screen, and some of the pictures were taken at 0 hr right after the injection.

Capt Vick: We have seen this phenomenon, however, with many other toxins. This is not unique to botulinum toxin, at least in our hands, and I hope it is not unique throughout the world, because we might feel uneasy. The snake venoms produce changes in cortical electrical activity much like that seen with botulinum, and, in addition, with certain venoms the change is irreversible. With others it cycles. We are now studying the injection of krait venom and copperhead venom, which produce marked depressions of cortical electrical activity, some recovery, and a subsequent loss.

Dr. Wills: I would like to give a brief summation by going back to the 10 questions, and if you look at the 10 questions on the board, you will find that all of the work presented in this conference has fallen in the regions of the 2nd, the 6th, and the 10th questions. None of the other questions have really been touched on except possibly a little about the third one, where the evidence is all obtained from in vitro experimentation.

SUMMARY COMMENT

Dr. Van M. Sim
Deputy Director of Medical Research
CRDL

For the past 2 days we have been discussing something that I think has been definitely needed in medicine. We have discussed possible mechanisms of action of toxins that are known to cause effects in animals and man. One of the problems of our "antibiotic era," starting with the sulfonamides, has been the lack of serious investigation of disease processes once a prophylaxis of therapy has been discovered. It seems appropriate to investigate an intoxication such as botulism, which has remained unsolved for at least 70 yr. Improved methods and more adequate techniques should produce new knowlege of value in other diseases affecting animals and man.

It is only possible, in summary, to comment on but a few of the contributions made during the past 2 days.

The part on which Dr. Lamanna made a statement some years ago in Science that the mouse has been the only animal even partially studied for the mechanism of action of botulinum toxin no longer holds true. There are many studies known to most of you, some from this laboratory and some from others, that were not reported here primarily because of time. The amount of work done here by Dr. Oberst and his group on the use of antitoxin, the use of assay materials, and methods of resuscitation has involved a great amount of effort and a great number of animals with reasonably inconclusive results. Nevertheless, we have profited from the negative findings, particularly in terms of guidance for our future reasearch programs.

In relation to the studies in comparative zoology, I do not feel at present that further studies on age and sex differentials and species differences in mouse and rats really pertain to the problem, because I think we have ample evidence on the mouse, rat, guinea pig, rabbit, dog, three species of monkey, and chimpanzee by the aerosol, intragastric, iv, im, and ip routes; it does not appear that the difference between the intranasal membrane and the gut lining is of particular importance in further studies of botulism.

I certainly think that work on gastric, pancreatic, and intestinal absorption is important. There has been much work done in the past on this.

Toxin has certainly been found to end up in thoracic ducts in fairly large concentrations. With the methods of labeling now available and with the possibility that there are means to reorganize fractions, headway can be made in studies of quantitation and localization of toxins or toxic fragments. We can gain some idea of how it is transported, particularly with the simultaneous studies made with light and the electron microscope. The work of Drs. Reisen and King is extremely important in relation to the fractions. This is not the first time we have heard of fractions but we are only beginning to understand that these things can be identified. It is not really surprising, I suppose, that a smaller molecule may be the active principle. Their relation to other properties, such as stability, will be important. In any event, laborious procedures, such as the use of iodine for labeling, should be very useful in the future.

It appears that localization of sites of action, as related by Dr. Brooks, has a better chance by use of microtechniques, not only for electrodes, but for biochemical analysis. Studies on the neuromuscular junction, the so-called "gutter," and, particularly, on the presynaptic portions are really indicated as basic work. We certainly must know more about the metabolism of the cells involved if and when we do find one or more groups of participating cells. The eye preparation of Mr. Wilson, known to some here, might be an appropriate biological preparation for some of this study.

The work of Drs. Sheff and Zack, utilizing a combination of biophysiological, biochemical, and morphological studies, was very interesting. The proposed 14-day preparations are very important. In review of Dr. Drachman's work, it seems reasonable that he does not propose that 40,000 MU of botulinum A is either prophylactic or therapeutic in its effect on chick embryos. It would be of great interest to know more of the characteristics and composition of fat in both the toxin-treated and denervated chicken. The massive dose used makes one wonder if this would not have happened if any neurotoxic substance had been used.

The work in hematology and specific organ enzymes is of interest but it is too early to estimate its application. Certainly, the kidney studies brought new evidence that requires further consideration. Comparative embryology in our heart-culture system may be of use in Dr. Rosenblum's work on isolated-heart preparations.

The utilization of latex coating by Dr. Covert may provide a means of more rapid diagnosis. Aberrant results might be obtained with people who have had horse-serum or transfusion reactions or have had previous liver disease or febrile illness.

In treatment, I'll mention very briefly the work done here and elsewhere. The work on photosensitivity reminds me very much of the work done in the early 30's on meningococcus and pneumococcus meningitis in which UV irradiation of blood was tried. They were usually nonspecific efforts; in some, dyes were added to blood to try to potentiate the effects. I am not aware of anything in the last few years being successful. At any rate, I think this work should continue; it is basic in relation to other information we desire and it probably will continue in-house. I am a little disturbed with the initial statement that if you do not give antitoxin within 3 min it is useless. This is not so; but I do not think the statement was made in the same context we are thinking about in relation to humans. If it is true, Dr. Tyler can correct me. I think the antiserum certainly is effective after longer periods of time. The biggest problem is the recognition of whether the patient has botulism and, second, to prepare to treat the patient rigorously, not only therapeutically but also with long term resuscitation as well as for all the complications from which most of the people seem to succumb.

Dr. Petty's program is intended to test the efficacy of human immune gamma globulin. Certainly, experience with other disease processes has demonstrated the feasibility. If these efforts are successful, we can turn our results over to USPHS for practical implementation.

I particularly want to thank Dr. Housewright, Dr. Schantz, Dr. Cardella, and Dr. Crozier and his group who have so willingly supported us through all these trying years when we had the responsibility for the program. Through the combined efforts of Detrick and Edgewood personnel, we have obtained a great deal of new information about the effects of Type A toxin in a variety of species by several routes of administration.

I think that in-house and with contractors, we still have the problem of rapid diagnosis. We must have a means of rapid diagnosis. We must have better treatment with a proved feasibility. We must also face the problem of mass prophylaxis in this country. So we must have a means of rapid diagnosis, better treatment, and it must be available. This all comes down finally to: Who is responsible? Is this a public health problem only? I think that with Fort Detrick, we have not only a moral responsibility, but, I hope, a continuing financial responsibility to carry on some of this work. In closing, I want to thank you again on behalf of Col Batte and Dr. Silver. I thank you all for participating. I think the presentations were excellent.

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CLOSING REMARKS

Col Joseph R. Blair
Director of Medical Research
CRDL

Gentlemen, I have not been able to attend all the meetings because of other meetings requiring my presence, and it was more appropriate that Dr. Sim be with you because he has been involved with botulinum toxin much longer than I have been. Dr. Wills has also been involved for a longer time than I, so I believe their presence was more necessary and useful than mine. Nevertheless, I have heard some of the sessions, and I was quite impressed with the quality of the presentations, both by the contractors and by our own people. I think you have done a splendid job and I want to thank you both for the laboratory and Edgewood Arsenal.

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13. ABSTRACT

Twenty monographs were presented at a conference on botulinum toxin held at Edgewood Arsenal on 28 and 29 June 1965. Each presentation was followed by a discussion period. Data in the following fields of research were presented: (1) pharmacological aspects, (2) relation to body weight, (3) dissociation of Type A toxin, (4) lethal subunits of Type A toxin, (5) availability of toxin forms, (6) important research questions, (7) possible absorption and distribution sites, (8) localization with lead binding at motor end plates, (9) isolation of muscle-tissue activity (10) fractionation and fluorescent labeling, (11) atrophy in chick muscles after massive doses, (12) photodynamic treatment, (13) pharmacodynamics, (14) clinical-pathological studies in monkeys, (15) the use of latex agglutination as a test technique, (16) results of IIT experiments, (17) production of human antibotulism serum, (18) lung absorption of high-molecular-weight molecules, (19) choline-C14 and acetylcholine C14 distribution, and (20) central and peripheral effects of Type A toxin. The basis for these studies was a close evaluation of the mechanism of action of the toxin, its effect on tissues, and an improvement in diagnostic and therapeutic techniques.

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14. KEYWORDS

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Choline C14
Central nervous system
Therapy

Proceedings
Botulinum toxins
Motor end-plate
Clinical studies
Respiratory paralysis
Acetylcholine C14
Autonomic nervous system