AD_____

(1)

RECEPTOR BINDING AND MEMBRANE TRANSPORT OF BOTULINUM TOXINS

ANNUAL REPORT

JOHN R. DANKERT

MARCH 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6062

University of Florida
Gainesville, Florida 32610

SECURITY CLASSIFICATION OF THIS PAGE A198925

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Florida; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Gainesville, Florida 32610	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-86-C-6062
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3M1-62770A871; TASK NO. AA; WORK UNIT ACCESSION NO. 386

11. TITLE (Include Security Classification)
(U) Receptor Binding and Membrane Transport of Botulinum Toxins

12. PERSONAL AUTHOR(S)
John R. Dankert

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 4/1/87 TO 3/31/88	1988 March	21

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	RA 1, protein/lipid interaction, artificial botulinum toxin inhibitor
06	15		
06	01		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The goal of this research is to characterize the interaction of botulinum neurotoxin with membranes, and seeks to add to the field of study of protein/lipid interactions in general. A model system of study has been developed to define which portions of the botulinum toxin insert and/or interact with artificial liposomes in order to define "membrane-active" portions of the molecule. This will enable a more defined study of the protein as it interacts with a bilayer through the use of protein and membrane probes to map the topography of the toxin in the membrane, and will also define possibly critical areas of the molecule which can be used as templates to produce artificial inhibitors of botulinum activity. (AUTH)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mary Frances Bostian	22b. TELEPHONE (Include Area Code): 301/663-7325	22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FOREWO:

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

Table of Contents

Figures

Tables

Introduction

The study of the mechanism of action of the botulinum toxins has centered upon the effect of the various types of toxin on living cells. The lethality of these protein molecules produced by strains of Clostridium botulinum is as yet unsurpassed by any other toxins, which makes the determination of their mechanism of action most important. The history of the study of botulinum toxins (botox) reveals the difficulties brought about by i) the dangers of working with such a poison, ii) the number of different serotypes of botox which can confuse the field of study, iii) the lack of precedence from other systems of protein toxins to produce working models that can be tested and applied to experiments on botox, and iv) the deficiency of suitable experimental procedures which have a small number of variables. This last item points to the difficulties of any studies performed on living cells in which innumerable processes are occurring simultaneously with the experiment.

Recently the study of botox has shifted to an interest in defining botox in molecular terms as opposed to phenomenological insights gained from work with living cells (1). This shift has been accompanied by advances in other systems of protein toxins which serve as models for the study of botox. The form of these advances in other systems is in the way protein molecules are viewed, specifically proteins which act similarly to botox in that they are toxins. These proteins are now seen to have some homologies in not only their end result (the impairment of function of a "target" cell), but also in a) the path(s) of uptake into the target and b) the structure of the protein molecules themselves. The structural similarities refer to a "domain" composition of these molecules, in which different segments of the polypeptide chain can be defined into various functional segments or domains (2,3).

The similarities above refer to paths of toxin uptake and of toxin activity. Surprisingly, the similarities in paths of uptake refers to a resemblance in uptake between eukaryotic and prokaryotic organisms. Both utilize a pH gradient formed in a sub-cellular space in which the toxin molecule is compartmentalized. For eukaryotic cells this is represented by acidic endosomes or lysozomes, both which form an acidic compartment in their interior through the use of proton translocating ATPases. Upon receiving the "signal" that they are near the their targets (i.e., the pH of the environment drops) a change occurs in the molecule which allows expression of its lethal activity. The expression of activity requires not only an enzymatic portion of the toxin molecule to act upon some internal structure(s), but also that the active portion of the molecule reaches this structure by crossing the endosomal/lysosomal membrane barrier. Crossing the membrane barrier has been shown to be dependent upon another domain of the molecule which allows the active domain access to the cytoplasm of the target cell. The generalized toxin molecule can the be divided into at least three separate functional domains: a) the enzymatically active portion, b) the domain which enables the active domain to cross the membrane barrier into the cytoplasm, and c) the binding domain of the molecule which functions to allow the toxin to bind to a specific receptor on the outside of the cell prior to internalization.

For prokaryotic cells and prokaryotic toxin molecules a similar situation is seen, as in the case of certain bacteriocins, molecules which are able to kill particular target bacteria. In the case of one class, that of the "E

colicins", the toxin molecule has been shown to contain at least three functional domains. The receptor binding domain which recognizes a specific protein (in this case the protein normally used by the bacterium to bind and transport a required nutrient, vitamin B12), the active domain which enzymatically acts to kill the target bacterium, and a domain which is required by the active domain for translocation across the bacterial cytoplasmic membrane (4,5). The analogy to eukaryotic systems is furthered by the fact that a drop in the pH of the environment of these colicins induces a conformational change in the protein. In the case of colicin E1, this change is accompanied by an increase in activity of a pore-forming ability in the molecule (6).

The similarities in these molecules which act as toxins spans the eukaryote/prokaryote barrier. Other resemblances include the high activity of these molecules. Although for the much larger eukaryotic cells more than one molecule may be required to kill a target cell trying to evade the toxic effect (note however that a determination of this number for eukaryotic cells has never been determined), in prokaryotic systems it has been shown that a single molecule of the toxin is capable of killing a target bacterium.

The molecular mechanism of action of these toxins may also be strikingly similar. For example, comparing the toxins colicin E2 and diphtheria, one finds that both work to inactivate protein synthesis of target cells (E. coli for colicin and mammalian cells for diphtheria). Both accomplish this task by ADP-ribosylating elongation factor 2.

In the case of botox, the mechanism of action is not known. However, using examples from other toxins and the processes of experimentation utilized it is our goal to elucidate the molecular mechanism of action of botox through two lines of investigation. They are 1) to determine the conditions necessary to develop a model system of artificial liposomes as targets for botulinum toxin action and 2) a continuation of this study to determine the portions of the molecule that are membrane inserting and to determine the orientation of the botox molecule in artificial phospholipid bilayers. The present report summarizes the findings of the interaction between botulinum toxin and artificial liposomes, followed by a discussion of future work. In addition, two of the most important aspects of this work have recently been initiated; the defining of the mechanism of action of this toxin and the development of an artificial inhibitor of the botox molecule.

Materials and Methods

Supplies. Sources for all chemicals and reagents for buffers and solutions unless otherwise stated were purchased from Fisher Scientific. Proteases used for digest studies were from Sigma, and dyes for the fluorescence studies from Molecular Probes, Inc. Lipids were obtained from Avanti Polar Lipids, and all electrophoretic chemicals were purchased from Serva. Botulimun toxin (Type B) was obtained from Calbiochem.

Lipid Vesicle Formation. Large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) were formed as described in (7,8).

Acid Shock Procedure. Vesicles in 10 mM Imidazole buffer containing 150 nM NaCl, pH 7 (10 µg lipid/mL) were subjected to a drop in pH by the addition of 10 µL of a 10% stock solution of succinic acid at pH 3.9. After 1 min the entire mixture was diluted into 3 mL of buffer at pH 7.4.

Fluorescence Measurements. Determination of release of the dye carboxyfluorescein (CF) from lipid vesicles was performed by forming the vesicles in the presence of a self-quenching concentration of the dye molecule (100 mM). The free dye molecules were separated from the internalized dye and liposomes by either passing the mixture over a Sephadex G-100 column (collecting the void volume fractions of the column for the vesicles) or by repeated centrifugations (at least 3 times) with resuspension into dye-free buffer. The vesicles were then used within 4 hours for all assays of toxin activity. Measurement of dye release was performed on an Aminco Bowman Spectrophoto- fluorometer, adapted with a thermostatted, stirred cuvette, as described in reference (8). pH shock was performed as described above using 10% succinic acid, followed by the addition of 10 µL of a 1 M Imidazole, pH 8 stock solution used to return the final pH to a value of 7.

Photolabeling Experiments. ^{125}I-TID (3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazarine) [Amersham] was used as a hydrophobic photolabel for the botulinum toxin molecule. After the photolabel was introduced into the reaction mixture, a small UV pencil lamp (Hammamatsu Corp., model number 81-1025-01) was then used to illuminate the mixture. The samples were kept totally free of light until UV illumination, and the illumination was performed in small open topped containers (Dynatech Laboratories, Inc., Immulon Removawell strips) with total volume not exceeding 100 µL. For the vesicle experiments, 10 µL of a 1 mg/mL final lipid concentration was placed in each well, with 1 µL of photolabel, and 10 µg of added protein. The pH drop (to pH 4) was performed as described above by the addition of succinic acid.

Gel Electrophoresis. SDS-PAGE gels of photolabeled botox were run as in (7) utilizing linear 10% gels with a 5% stacking gel. The completed gels were then sliced into vertical strips for each lane containing samples, and these vertical strips were then cut horizontally every 5 mm. The horizontal 5 mm pieces were then placed individually into test tubes and assayed for radioactive counts on a Beckman gamma-counter. The 5 mm pieces were labeled as "zones" of the gel which could be compared to the mobility of stained molecular weight standards run on the same gel. In this way the effective mobility of the radioactive counts in each zone could be assigned a molecular weight range.

For the Figures, Zones 1 through 11 correspond to the molecular weight (i.e., relative mobility) ranges of: 1= >300 kD; 2= 240-300 kD; 3= 180-240 kD; 4= 120-180 kD; 5= 90-120 kD; 6= 70-90 kD; 7= 50-70 kD; 8= 30-50 kD; 9= 10-30 kD; 10= <10 kD and 11= bromphenol blue dye front.

Results

The ability of the botulinum toxin molecule to alter the permeability of a lipid bilayer was investigated through the use of dye-encapsulated liposomes and liposomes with an artificially induced membrane potential. For the dye leakage experiments, vesicles with carboxyfluorescein (CF) inside the vesicles were placed into a stirred, thermostatted cuvette of a fluorometer. The level of fluorescence was monitored continuously over the course of the experiment. Shown in figure 1 is the fluorescence of the CF-encapsulated liposomes showing the stability of the integrity of the bilayer over the course of 1 hour. Also shown is the large increase in fluorescence after the addition of an agent which causes the permeability barrier of the liposomes to collapse, the detergent Triton X-100. The rapid increase in signal is at the limit of detection of the monitoring fluorometer, and occurs within 1-2 seconds after detergent addition. Since the effect of a pH shock on the toxin was to be investigated, it was necessary to determine the response of the vesicles themselves to a pH shock (pH 4 for 1 min). Table 1 summarizes the effect of this acid shock on liposomes composed of a variety of compositions. Note that all of the vesicles that contained a negatively charged phospholipid phosphatidyl serine were subject to some decrease in permeability after restoration of the pH to 7. This is probably due to the formation of hexagonal forms of the lipid (due to "titration" of the acidic phospholipids with protons at low pH) which would cause large holes and gaps in the vesicles, or even to complete loss of bilayer structure. The dependence on phospholipid chain length was also found to be critical, in that the DMPC/DOPC vesicles were much more sensitive to this shock as compared to DPPC/DOPC vesicles (14 carbons for DMPC, 16 for DPPC). Table 1 also shows that 5% cholesterol added to the DPPC/DOPC vesicles led to a further stabilization of bilayer structure during the acid shock procedure.

The DPPC/DOPC/Cholesterol (45%/45%/5%) vesicles were then used for subsequent studies on the botulinum toxin molecule. When the molecule was added to vesicles at pH 7, no change in fluorescence was seen (Fig 2), and is consistent with earlier reports on the ability of botox to form channels in supported lipid bilayers. When isolated toxin was subjected to an acid shock, returned to pH 7, and added to vesicles no increase in fluorescence was seen indicating that the bilayer structure had not been compromised under these conditions (Fig 2.). Figure 3 shows that when the toxin molecule was present with the vesicles only at the time of the acid shock an increase in fluorescence could be seen. This is similar to other studies on different toxin molecules such as diphtheria (15), colicin E1 (16), and the membrane-active protein of the human complement system, C9 (12). This ability of botox to alter the permeability barrier could not be duplicated when the toxin protein was heated for 5 min at 100°C (data not shown).

The membranolytic activity of botox was further characterized through the use of photoaffinity labeling. The hydrophobic photolabel, ^{125}I-TID (3-(trifluoromethyl)-3-(m-[125-I]iodophenyl)diazarine) was used to determine the extent of toxin penetration into the lipid bilayer, and also as a basis for future studies to determine the actual portions of the molecule that are embedded into the membrane bilayer. In this assay, the photolabel was pre-incubated with the vesicles, and this label partitioned into the hydrophobic interior of the vesicle bilayer. The toxin molecule or other protein of interest was added (with or without the pH shock) and the mixture was then

illuminated with a high intensity UV light source for 1 min. The mixture was then processed for SDS-PAGE and autoradiography as described in Methods.

Shown in Fig. 4 are the results of the measurement of radioactivity in slices from the gel lanes performed as in Materials and Methods. Botox alone with the photolabel shows under non-reducing conditions a major peak (120 to 180 kD). This corresponds to the reported molecular weight of 145,000 of this botox type (1). When the toxin was subjected to an acidic pulse prior to photolabeling, an additional high molecular weight aggregate is formed that has an apparent molecular weight of over 300,000 (Fig. 4). The reason for the appearance of this high molecular weight aggregate is not known, but may indicate that some pH-dependent conformational change has occurred which causes the protein to form SDS-resistant polymers.

Botox was then subjected to photolabeling in the presence of artificial vesicles. Using vesicles that are not by themselves rendered permeable by an acid shock nor became permeable when acid-shocked in the presence of botox (using vesicles composed of 100 % DPPC, from previous data, shown in Table 3, Annual Report 1987) it was seen that the toxin molecule was not labeled to any significant extent when irradiated (UV light) with or without an acidic pulse prior (30 sec) to the irradiation (Fig. 5).

Vesicles that were subject to CF release only when acid shocked in the presence of botox were then examined. In this case (Fig. 6) it is seen that, in addition to the labeling of the phospholipid molecules seen as the counts in zone 11, an increase in labeling of the 120 kD to 180 kD zone can be detected. Glutathione (GSH) is a small molecule that is soluble that can act as a "sink" for this photolabeling procedure, and can be used to distinguish between proteins that are surface associated (or weakly bound to the vesicles) from those that have segments which are buried in the hydrophobic core of the bilayer. GSH was added at a concentration of 25 mM which is at least 1000 times in excess of botox, and should dilute the portion of the photolabel that botox receives if it is not buried in the bilayer (9). Figure 6 shows that GSH did reduce the labeling of the toxin molecule, but did not completely eliminate the labeling. It should be noted that the majority of the label on the toxin appeared in a lower molecular weight peak (≈70 to 90 kD zone) and is caused by the ability of GSH at such a high concentration to effectively act as a reducing agent. This probably represents label that is incorporated into the heavy chain of the botox molecule (1). The reduction in the amount of label that was seen in the presence of GSH could be due to a decrease in the labeling of those portions of the molecule that are near the vesicular surface at the time of irradiation, and that portion of the molecule that is labeled represents a portion(s) of the toxin that is buried in the membrane.

The ability of the botox molecule to be tagged by the hydrophobic photolabel, TID, has been done under a variety of conditions of acid shock, vesicle composition, and presence or absence of GSH. These results are summarized in Table 2. It can be seen that the toxin molecule can only be effectively labeled after an acid shock treatment. It should be noted that when the labeling of the toxin was performed with vesicles composed such that acid shock alone causes a breakdown in their permeability (i.e., DPPC/PS), GSH could not consistently decrease the amount of label incorporated into the toxin molecule. It is possible that GSH can no longer act as a sink for label (or is less efficient as a sink) due to rearrangement of the phase structure of the phospholipid molecules which could act to further bury the toxin molecule into hydrophobic regions that are not accessible to the water soluble GSH. Since

this induced change is independent of the toxin molecule, we feel that any portions of the protein that may be labeled under these conditions is not relevant to the mechanism of action of the botox molecule.

Recently we have begun using the acid-pulsed mixture of membrane vesicles and botox to determine which portion of the molecule are accessible to proteases. The enzyme in the aqueous phase will not be able to digest those portions of the toxin that are either inside the lumen of the liposome or buried in the hydrophobic interior of the bilayer. Digests with the enzyme trypsin on the DPPC/DOPC/CHOL vesicle - Botox mixture (after acid shock treatment) have been complicated by the low yield of protein after treatment (data not shown). The trypsin is effective at digesting the botox before acid-shock in the presence of vesicles, however, and therefore we are currently establishing a procedure to label the toxin embedded in the vesicles (with ^{125}I) in order to detect small proteolytic fragments from the digests on SDS-PAGE gels followed by autoradiography.

Discussion

The finding that botulinum toxin can disrupt the integrity of the membrane bilayer of artificial vesicles when subjected to a low pH pulse (pH 4 for 1 min) for certain artificial liposomes can be used as to further characterize the in vivo action of the toxin. Similarly, as the toxin has been shown here to insert into the hydrophobic interior of these vesicles, the actual portions of the molecule that are buried in the membrane may be sought, and that future studies using proteolytic agents on the photolabeled botox molecule can determine this. The finding that some vesicle compositions that are disrupted by the acid shock alone yet do allow the photolabel to be introduced into toxin molecule is of most importance. Further studies on determining the location of the photolabel along the peptide backbone of the toxin molecule could be complicated by this non-specific, and that a true picture of toxin orientation in the bilayer must be performed under conditions that maintain the integrity of the bilayer in the absence of toxin.

Recent work in two other areas of research have directed our present studies towards the determination of i) the specific mechanism of action of the toxin and, most importantly, ii) to the development of an artificial inhibitor of the botulinum toxin molecule. The first of these two goals has come about through the demonstration of common protein sequence antigens in diverse toxin-like molecules and the second through recent advances on inhibitors of small hormone molecules in the human body.

Concerning the mechanism of action of the toxin, it has recently been shown by one of us (10) that two diverse proteins that have been shown to be membranolytic, namely the human complement component C9 and a component of bee venom, melittin, share common sequences of amino acids, act as antigens for each others antibodies, and antibodies to melittin have been shown to inhibit the lytic activity of C9 (10,11). This implies that these two proteins share a similar mechanism of action, even though the size (538 amino acids for C9, 26 for melittin) and source of the proteins are so different. Taking this into account we have begun to initiate the production of antibodies to portions of the botox molecule. As our studies with the photolabel point to certain membrane embedded region(s) of the botox molecule as being important for membrane interaction we have started with this segment(s) of the protein. As almost all of the toxins grouped into the "generalized toxin molecules" (colicins, C9, diphtheria, etc.) require a membrane-spanning region and others contain, in addition, a membrane-active portion which actually causes the toxic effects to be manifested (4,12) we feel that the development of antibodies to this region(s) will be useful.

In addition we have begun obtaining antibodies (polyclonal and monoclonal) to many of the "generalized toxin molecules". These antibodies will then be used for cross-reactivity tests with botox to determine similarities in sequence (as yet undefined for botox) and function. Through these studies it may be possible to define the regions of the molecule responsible for lethal activity. Any common cross reactivities found could point to similarities in any one of the three generalized functions of these toxins: i) the enzymatic unit, ii) the membrane inserting or translocating domain, and iii) the receptor binding domain.

The development of an artificial inhibitor for the botox molecule may soon be the most important part of this research effort. It has recently been shown that small peptide hormones may be influenced by artificial peptides

manufactured according to one of two methods. One involves the generation of a peptide from the opposite strand of DNA that encodes the hormone peptide. Using this approach it was shown that the activity of the hormone ACTH could be inhibited by a peptide manufactured as if the "opposite strand" message was translated in protein (13). This inhibitor ("HTCA") probably acts as an artificial receptor for ACTH and therefore acts as an inhibitor to the hormonal effects of ACTH. Also, a novel method to produce similar inhibitory peptides is being proposed for other peptide hormones. In this case, arginine vasopressin (AVP) is being used as a model to demonstrate the feasibility of producing artificial inhibitory peptides to this hormone. This method involves the use of an algorithm which bases selection of the "inhibitor's" amino acid sequence loosely upon pairing of hydrophobic and hydrophilic residues between AVP and the inhibitor (14). This technique is currently being patented by Dr. Howard M. Johnson in our department at the University of Florida, and he has given our lab advanced access to his algorithm. Currently we are arranging to have our hydrophobically-labeled sequences of the botulinum toxin molecule sequenced. Since this new algorithm does not require the DNA sequence (to produce the protein of the opposite strand) to be known, we will not be dependent on obtaining this information, but rather only on our ability to sequence portions (amino acids) of the molecule.

The reported ability of these artificial inhibitors provides a unique opportunity to produce an agent that can counter-act the effects of the botulinum toxin. The ability of these molecules to inhibit the expression of activity of small hormone molecules demonstrates their capability. If molecules as small and as effective as these can be inhibited one could argue that a similar inhibition could be obtained for larger molecules. Since we are defining important regions of the botox molecule only artificially produced inhibitors to these relatively small areas will be needed. If it is possible to produce such an inhibitory peptide, the normal route of production of the body's own defense (that of antibody production to the toxoid) which takes weeks or months to take effect will not be required, or, may provide an antidote in emergency situations until an effective antibody titre is produced. Our ability, in utilizing the defined artificial liposome system, to monitor i) the binding of botox to membranes, ii) the insertion of regions of the molecule into the bilayer, and iii) the dissipation of ion gradients by the toxin enable us to determine how any artificial inhibitor is able to inhibit botox activity. Indeed, any such inhibitor produced, even if never used as an "antidote" will give us insight to the mechanism of action of botox if the activity of the molecule can be selectively inhibited.

In summary, future work on our study of the botox molecule will include a complete characterization of the regions of the botox protein that are embedded in the artificial membrane bilayers and to determine how other factors (i.e., pH shock) are able to allow expression of this activity. In addition, a search for common antigenic and <u>functional</u> sights between botox and other molecules that have a "membrane-activity" has begun. It is hoped that this will allow us to predict the mechanism of action of the botox molecule and to define the segments of the molecule involved in this activity (which are most likely distinct from the regions buried in the membrane). Finally, protein sequencing of the membrane embedded portion(s) of the molecule has begun (and any regions of the molecule found to show a common binding site to known toxin molecules will also be sequenced) in order to produce possible inhibitors to the botox molecule.

Table 1. Effect of Acid Shock Procedure on Liposomal Membrane Integrity.

Composition (%)	% Dye Release
Asolectin	55 ± 13
Asolectin/CHOL (95/5)	27 ± 9
DMPC (100)	83 ± 15
DMPC/DOPC (50/50)	66 ± 10
DMPC/DPPC (50/50)	42 ± 8
DMPC/DPPC/CHOL (48/47/5)	29 ± 7
Egg PC (100)	9 ± 3
Egg PC/DPPS (70/30)	69 ± 12
DPPC (100)	6 ± 3
DPPC/DPPS (70/30)	97 ± 18
DPPC/DOPC (50/50)	6 ± 2
DPPC/DOPC/DPPS (35/35/30)	53 ± 8
DPPC/DOPC/CHOL (48/47/5)	2 ± 1
DPPC/DOPC/CHOL/DPPS (35/35/5/25)	37 ± 6

Table 2. Ability of Botulinum Toxin (type B) to be Labeled by the Hydrophobic Radioactive Tag, ^{125}I-TID.

Composition	GSH	% Incorporation
Egg PC	-	45
Egg PC	+	35
Egg PC/DPPS	-	63
Egg PC/DPPS	+	47
DPPC/DOPC/CHOL	-	77
DPPC/DOPC/CHOL	+	52
DPPC	-	8
DPPC	+	6
DPPC/DPPS	-	78
DPPC/DPPS	+	75
DPPC/DOPC	-	34
DPPC/DOPC	+	25

% Incorporation of total counts not including counts in the dye front (i.e., free label and labeled phosopholipids).
Data expressed as average of 3 expts, error = +/- 10%.

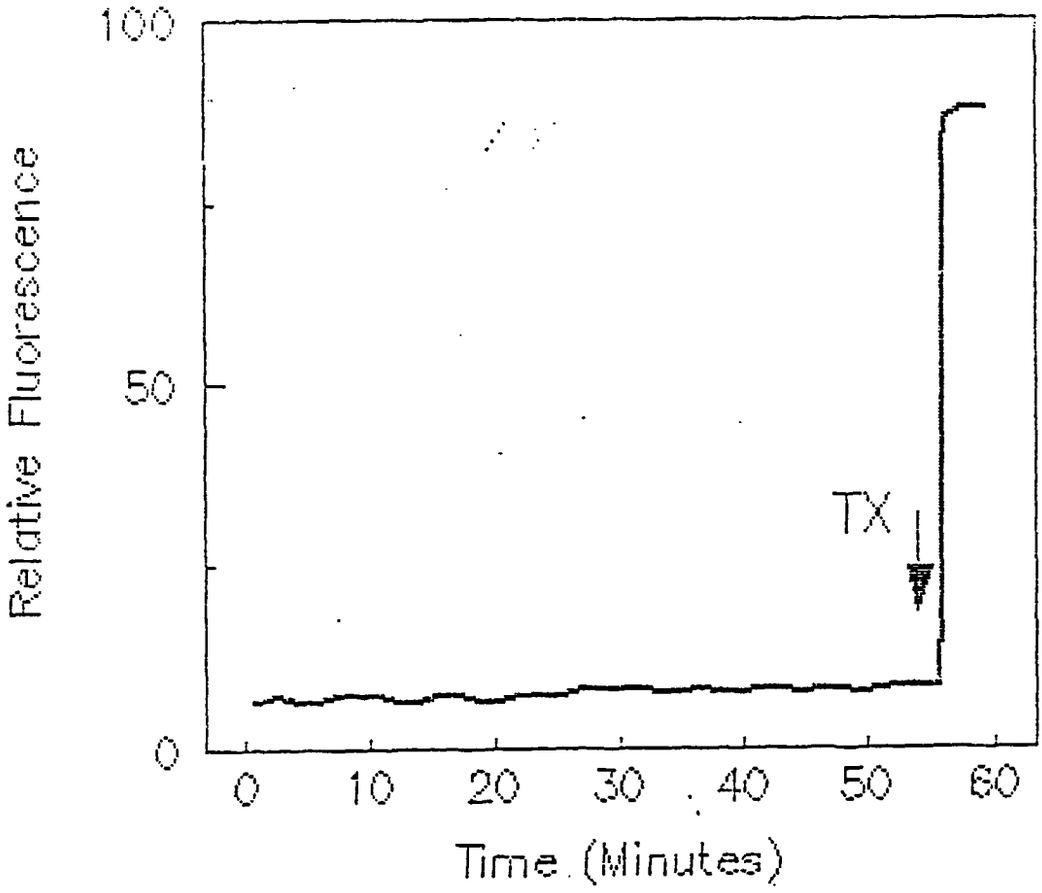

Figure 1. Stability of the fluorescence of Carboxyfluorescein (CF, 200 mM) in lipid vesicles (DPPC/DOPC/Cholesterol, 48%/47%/5%). 10 µg of lipid was diluted 100 X into 2 mM immidazole, 150 mM NaCl, pH 7 in a stirred thermostatted cuvette (37°C) of a spectrophotometer and the fluorescence at 520 nm was monitored (excitation = 475 nm). At the indicated time (TX) the detergent triton X-100 was added to a conc. of 0.1%.

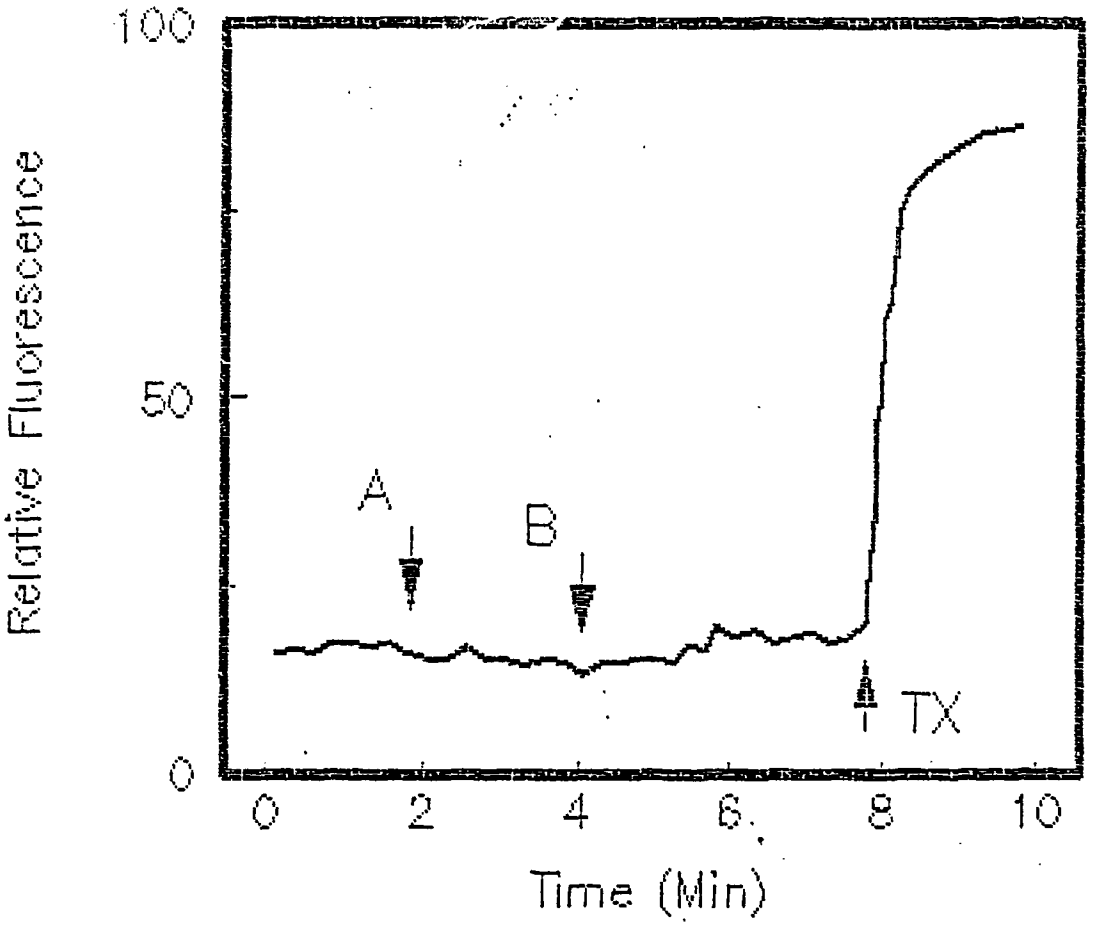

Figure 2. Inability of Botulinum toxin or acid treated toxin to induce the release of CF from liposomes. Conditions as in figure 1. A = addition of .05 µg Botulinum toxin type b. B = addition of .05 µg of botulinum toxin that had been acid-shocked prior to addition to the liposomes (to pH 4 for 1 min by addition of 10% succinic acid, pH 3.9 followed by addition of 1 M imidazole, pH 7). The botox was used within 10 min and kept on ice.

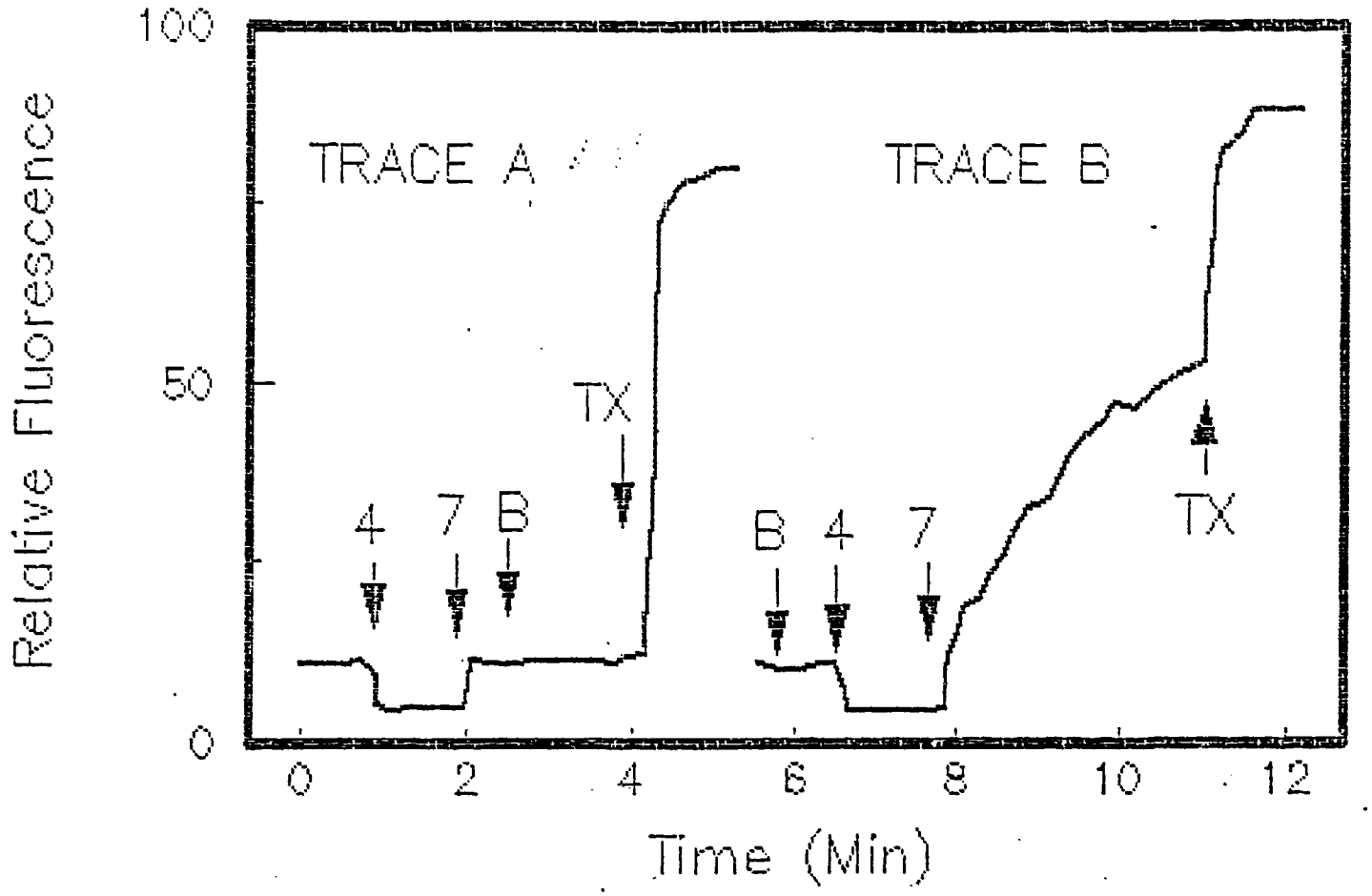

Figure 3. Ability of Botulinum toxin to increase the permeability of liposomes in the presence of an acid-shock. Conditions as in figure 1. At the arrows labeled 4 and 7 the pH of the liposome suspension was made either pH 4 or pH 7 respectively as described in figure 2. B = addition of .05 µg of botulinum toxin, TX = triton X-100 added to 0.1%.

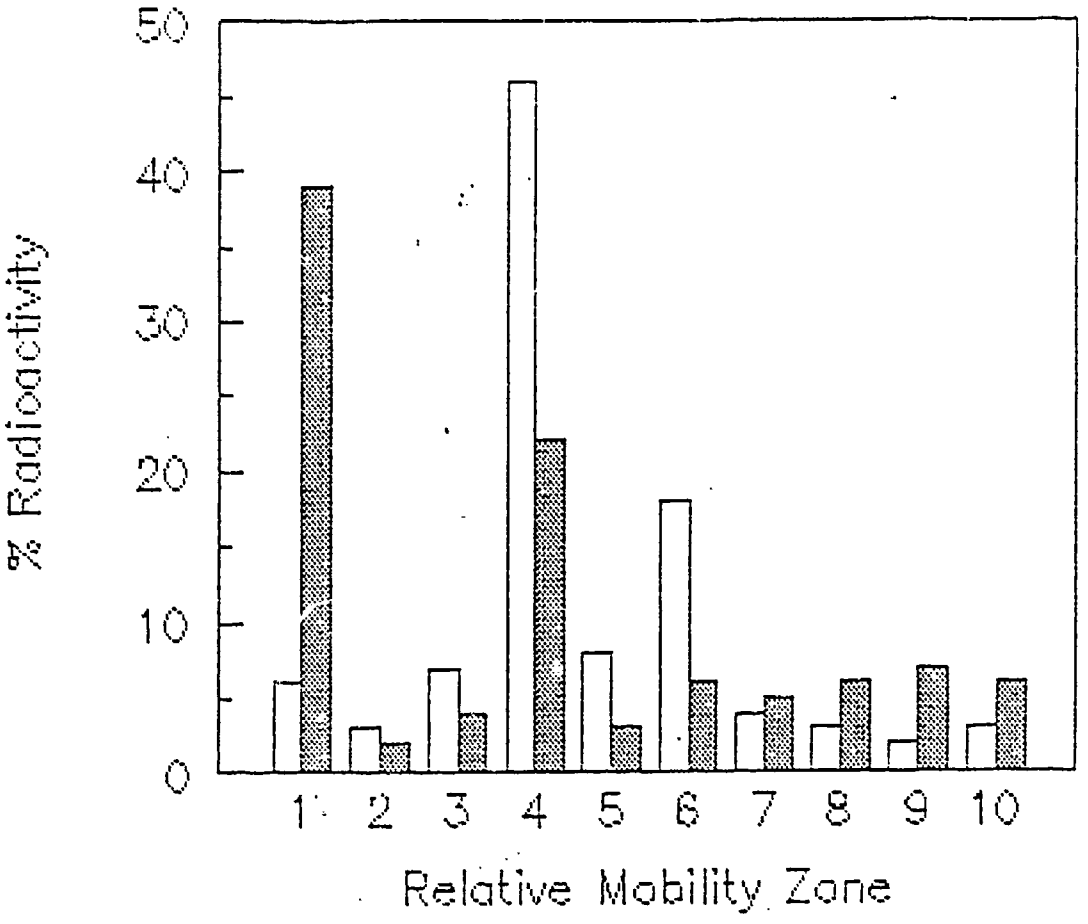

Figure 4. Photolabeling of Botulinum toxin before and after acid-shock. Botulinum toxin (1 µg) was photolabeled with TID in the absence of liposomes, separated by SDS-PAGE, and the gel cut into pieces for counting as described in methods. Open bars = toxin labeled at pH 7, closed bars = toxin labeled after an acid-shock as described in figure 2. % Radioactivity = relative counts in each gel piece. Relative mobility zone corresponds to the molecular weight ranges as described in Methods.

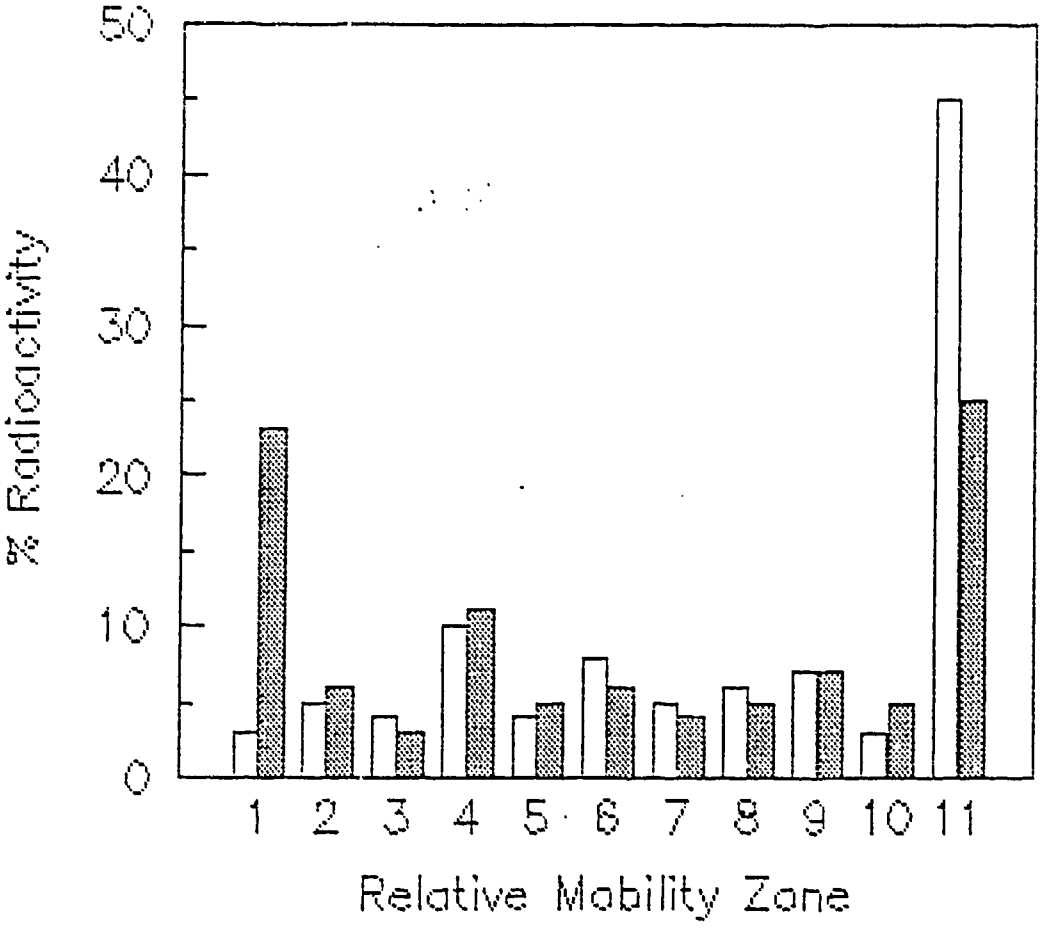

Figure 5. Photolabeling of Botulinum toxin by the hydrophobic photolabel TID in the presence of DPPC liposomes. DPPC liposomes that are not subject to botox-induced (with acid-shock) leakage were used for the experiment. Lipid to protein ratio (M/M) = 100 to 1. Open bars = photolabeling performed at pH 7, closed bars = photolabeling was performed 30 sec after an acid-shock to pH 4 for 1 min with return to pH 7. Other conditions as in figure 4.

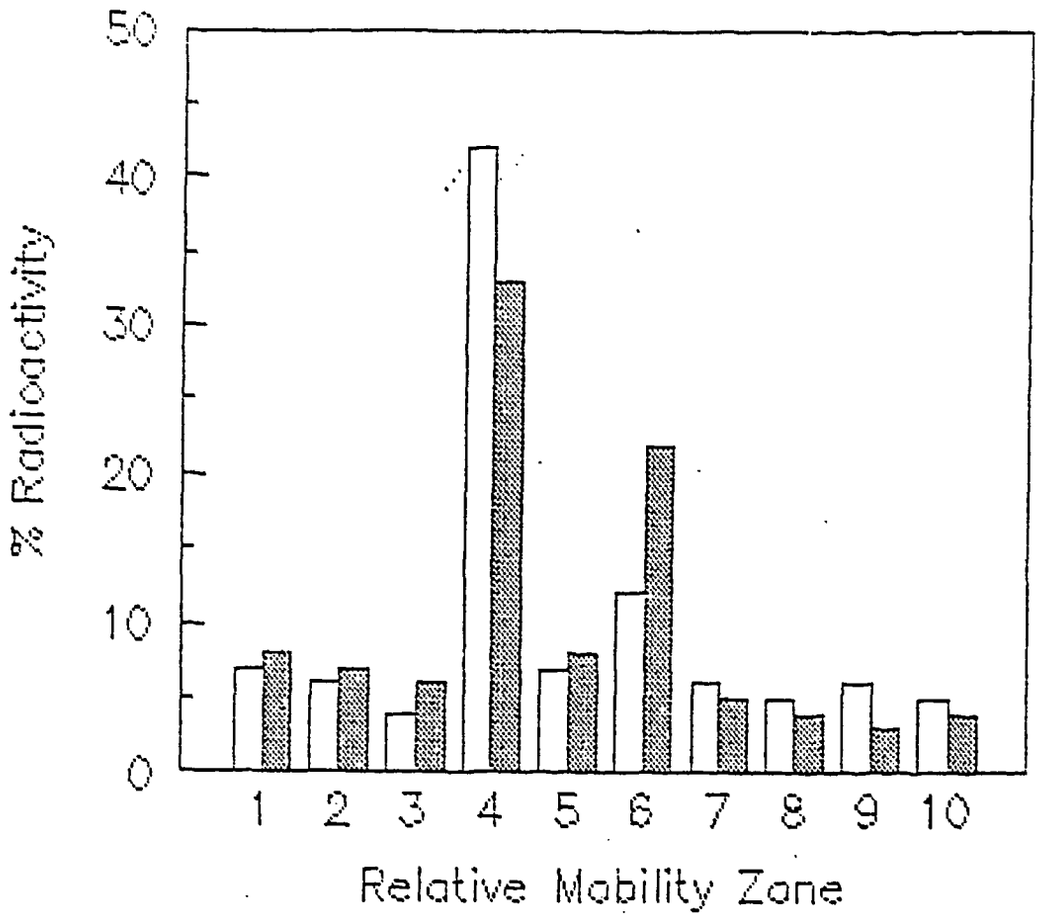

Figure 6. Photolabeling of Botulinum toxin by the hydrophobic photolabel TID in the presence of botulinum-sensitive (with acid-shock) liposomes. Liposomes were composed of 48% DPPC/47% DOPC/ 5% Cholesterol. Both open and closed bars subjected to acid-shock as described in figure 5, with the closed bars representing photolabeling in the presence of 25 mM Glutathione. For this figure, relative counts are presented not including those counts in the dye front (gel zone 11).

References

1. Simpson, L.L. (1986) Ann. Rev. Pharmacol. Toxicol. 26:427.
2. Habermann, E. and F. Dreyer. (1986) Curr. Top. Micro. Immunol. 129:93.
3. Hoch, D.H., M. Romero-Mira, B. Ehrlich, A. Finkelstein, B. DasGupta, and L. Simpson. (1985) PNAS (USA) 82:1692.
4. Dankert, J.R., Y. Uratani, C. Grabau, W.A. Cramer, and M. Hermodson. (1982) J. Biol. Chem. 257:3857.
5. Collier, R.J. (1977) IN: The Specificity and Action of Animal, Bacterial and Plant Toxins, ed. P. Cuatrecasas, p. 67, Chapman and Hall, London.
6. Bullock, J.O., F.S. Cohen, J.R. Dankert, and W.A. Cramer. (1983) J. Biol. Chem. 258:9908.
7. Dankert, J.R., J.W. Shiver and A.F. Esser. (1985) Biochemistry 24:2754.
8. Shiver, J.W., J.R. Dankert, J.J. Donovan and A.F. Esser. (1986) J. Biol. Chem. 261:9629.
9. Brunner, J. and G. Semenza. (1981) Biochemistry 20:7174.
10. Laine, R.O., B.P. Morgan and A.F. Esser. (1988) Biochemistry (in press).
11. Laine, R.O. and A.F. Esser. (1987) Meeting of the 12th International Complement Workshop, Abs. 157.
12. Dankert, J.R. and A.F. Esser. (1987) Biochem. J. 244:393.
13. Blalock, J.E. and K.L. Bost. (1986) Biochem. J. 243:679.
14. Johnson, H.M. and B.A. Torres (1988) J. Immunol. (Submitted).
15. Shiver, J.W. and J.J. Donovan. (1987) BBA 903:48.
16. Shiver, J.W., W.A. Cramer, F.S. Cohen, L.J. Bishop and P.J. deJong. (1987) J. Biol. Chem. 262:14273.

Distribution List

5 copies

Commander
US Army Medical Research Institute of
Infectious Diseases
ATTN: SGRD-UIZ-M
Fort Detrick, Frederick, MD 21701-5011

1 copy
~~4 copies~~

Commander
US Army Medical Research and Development Command
ATTN: SGRD-RMS-M
Fort Detrick, Frederick, Maryland 21701-5012

2 copies

Defense Technical Information Center (DTIC)
ATTN: DTIC-DDAC
Cameron Station
Alexandria, VA 22304-6145

1 copy

Dean
School of Medicine
Uniformed Services University of the
Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799

1 copy

Commandant
Academy of Health Sciences, US Army
ATTN: AHS-CDM
Fort Sam Houston, TX 78234-6100