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SITE-SPECIFIC ANTAGONISTS TO TETRODOTOXIN AND SAXITOXIN

ANNUAL REPORT

C. Y. KAO

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13. ABSTRACT *(Maximum 200 words)*

Towards the aim of developing rationally designed site-specific antagonists to tetrodotoxin (TTX) and saxitoxin (STX), the physical dimensions of the binding site and some key anchoring site-points in it for TTX and STX have been deduced from studies of structure-activity relations of analogues of these toxin molecules. The binding site is probably a pocket in the sodium-channel protein 9.5 A wide, 6 A tall, and 5 A deep, containing one ion-pairing and 4 hydrogen-bonding sites common to both TTX and STX. There are 1 or 2 sites unique to either toxin. If glutamate 387 of rat brain sodium channel were taken as the anionic site a of this work, then the carbonyl oxygen of asparagine 388 would be the hydrogen-bonding sites b and a.

We have oxidized TTX to 11-oxoTTX, an important synthon for other TTX derivatives. From it, we have made a specifically labelled ^{3}HTTX of high specific activity, which is being used in attempts to locate the TTX binding site directly. Based on our present knowledge of the TTX/STX binding site, other compounds are now being synthesized to attempt to bind to some of the identified site-points for possible use as antagonists to TTX and STX.

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INTRODUCTION

Project goals. The objective of this project is to generate more knowledge about the specific chemical structures of the tetrodotoxin (TTX)/saxitoxin (STX) binding site on the voltage-gated sodium channel protein. It is hoped that from such knowledge, site-specific antagonists to these toxins can be developed rationally. Moreover, identification of the binding site will greatly aid further understanding of the three-dimensional structure of the sodium-channel, and such knowledge will facilitate our understanding of the actions of other sodium-channel effectors, and the developemtn of appropriate specific antagonists.

The project has been progressing on two parallel tracts: (a) to expand and refine current knowledge of the structure-activity relations of TTX/STX analogues, and (b) to produce new synthetic compounds which might mimic or block the actions of TTX/STX by interacting with the TTX/STX binding site. On track (a) the work consists largely of electrophysiological studies of newly discovered natural analogues of TTX and/or of STX, utilizing the voltage-clamped preparation to study specific ionic conductances. This phase of the work is now virtually complete. All reactive groups on the suface of the TTX molecule, and all but the C-10 groups of the STX molecule have been touched through the examination of at least one representative analogue. Such refinements during the last contract year have led us to formulate the probable physical dimensions of the TTX/STX binding site, with 5 - 6 anchoring points for specific reactive groups in the toxin molecules. Further, we have identified two amino acid residues which could account for 3 of those anchoring points.

On track (b), past attempts to synthesize new compounds have been hampered by the limited knowledge of potential reactive binding sites. Because of the new developments in track (a), we are taking new approaches to this work. We have also made and isolated the first reactive derivative of TTX, and from it, we have succeeded in producing a specifically labelled radioactive TTX of high specific activity. This labelled compound will now permit us to go directly after the TTX/STX binding site through appropriate biochemical and peptide-mapping studies.

Background. Tetrodotoxin (TTX) and saxitoxin (STX) are important neurobiological tools because of their specific reaction with the voltage-gated sodium channel. They are among the most lethal, low molecular-weight, non-protein toxins known, each with a LD_{50} of 10 ug/kg body weight. They are very different in chemical structures, but their biological actions are virtually identical.

Although they came into use in the 1960's, the chemical basis of their action remains unclear. The difficulty is attributed chiefly to obstacles in studying the chemical properties of these molecules. For example, TTX is practically insolble in any solvent except slightly acidified water. Early attempts at modifying the structure of TTX generally led to marked loss of biological activity. For STX, uncertainties about its structure persisted into the early 1970's. Neither toxin molecule has chromaphores in the UV or visible spectral range, making their detection a formidible task. However, because of advances in separation technology, natural analogues of both TTX and STX were discovered in the mid 1970's. Moreover, postcolumn-derivatization procedures led to sensitive detection methods which required rather minute amounts of material. Many of the natural analogues turn out to be modified in small and discrete manners, and to retain some biological activity. By studying the structure-activity relations of some of these analogues, three groups in each molecule were recognized as being crucial for activity, and to

be placed in rather similar stereochemical positions. These groups are:

TTX	STX
1,2,3 guanidinium	7,8,9 guanidinium
C-9 hydroxyl	C-12 hydroxyls (gem-diols)
C-10 hydroxyl	

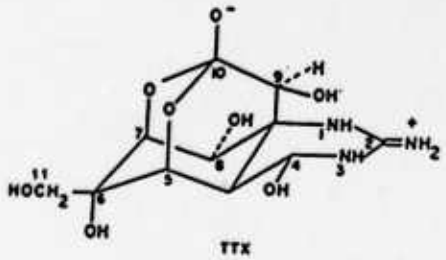
These groups form the initial, identified active portions of the toxin molecules. In my conception, the guanidinium groups formed ion-pairs with site-points in the receptor, whereas the hydroxyl groups formed hydrogen-bonds. In new work done on this project, an additional ion-pairing site has been found for an analogue of STX, 3 additional hydrogen-bonding site-points have been found for TTX, of which 2 appear to be shared in common with STX. Some aspects of this work have been reported in previous annual reports in preliminary form. Because our thoughts are more clearly crystallized, I will submit a proposed model of the TTX/STX binding site for consideration below.

It should be recognized that such a proposal is only a hypothesis which needs to be tested. For such purposes, we have made a reactive derivative of TTX which is 5 times more potent than TTX itself. From this derivative, we have produced a specifically labelled radioactive TTX of high specific activity. We expect to be using this unique radiolabel to conduct peptide-mapping of the binding site.

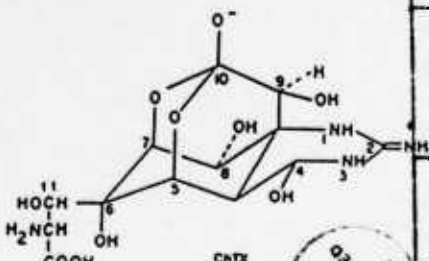
WORK DONE IN THE PAST YEAR

Electrophysiological Work

6-epi TTX, 11-deoxy TTX, and Chiriquitoxin (ChTX). These compounds ae natural analogues of TTX. The first two were found in an Okinawian newt, Cynops ensicuda (1); the last in a Costa Rican frog (2). I have reported on these compounds in previous quartery and annual reports, but because of their relevance to the formulation of the TTX/STX binding site, I will briefly restate the most salient features of their properties and actions. Their structures are shown below, in comparison with that of TTX.



TTX



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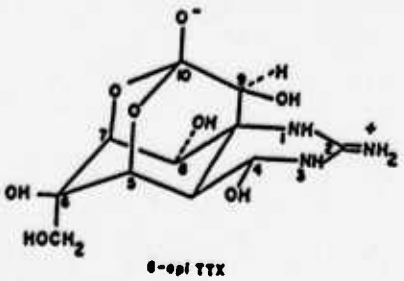
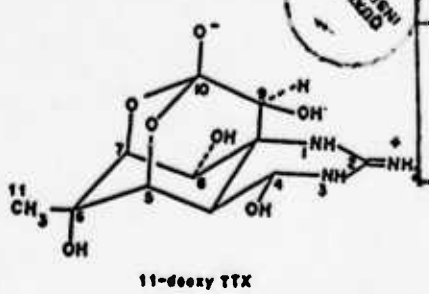


6-epi TTX



11-deoxy TTX

In 6-epi TTX, the hydroxyl and hydroxymethyl groups on C-6 of TTX are in an epimeric configuration. In 11-deoxy TTX, the hydroxymethyl group on C-6 is changed to a methyl function. In chiriquitoxin (ChTX), a methylene hydrogen of the C-11 hydroxymethyl function is replaced by a glycine group. 6-epi TTX, with the least structural alteration, has only 3% the potency of TTX in blocking sodium channels. 11-deoxy TTX has only 1% the potency. ChTX, with the largest structural alteration, has 100% of the potency. The results have been interpreted to mean that the -OH groups on both C-6 and C-11 are important for activity, but the lack of any influence of the large glycine moiety in ChTX seems like a puzzle. A reasonable resolution will be discussed below.

All of these compounds also affect the potassium channel in a distinctive way. They slow the rate of activation of the fast potassium current. The effect is potentially important, and much effort was spent in the past year to see whether there was any relation to the sodium-channel blockage. The effect is rather variable from muscle fiber to muscle fiber, is unrelated to the concentration of toxin applied, and can be seen in sodium-free media and when sodium current is either inwards or outwards. Significant slowing of the fast potassium current is seen in about 40% of the muscle fiber population in cases of TTX, 11-deoxyTTX and ChTX, but not 6-epiTTX or norSTX. We finally concluded that this potassium channel effect is unrelated to the sodium-channel blockage, but was possibly assoicated with the configuration of the groups on the C-6 position.

8-hemisuccinyl TTX. This compound is a synthetic derivative which has not been studied before. Dr. Mari Yotsu, one of our colleagues in Japan, unexpectedly produced this compound (unpublished information). The reaction is expected to occur on the C-11 alcohol function, but structural determinations confirm that succinylation had occurred on the C-8 -OH which no one had found a way to manipulate. We assayed the compound and found it to be 4% as active as TTX. With this analogue, all the surface active groups (mostly -OH's) of TTX have been touched in our structure-activity studies.

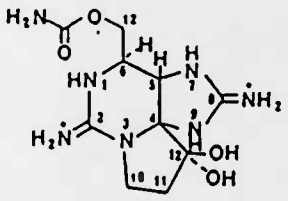
High purity TTX. Dr. T. J. Huang of the Hebei Fisheries Research Institute in China supplied this compound for us to assay. That institute has produced a commercial TTX product which we have found to be identical and perhaps slightly better than the TTX from the Japanese company Sankyo (3). The original Hebei sample, like the Sankyo TTX, has about 10 - 15% anhydroTTX. Since anhydroTTX is only 1% as active as TTX in blocking sodium channels, that amount of contamination is not important in biological experiments. The new High Purity TTX contains no detectable anhydroTTX (unpublished NMR data of H. S. Mosher), and is therefore chemicaly of higher purity. On assay on the frog skeletal muscle fiber, however, we were surprised to find it about twice as potent as ordinary TTX. Since there is no good chemical reasoning for this observation, we suspect some error in the concentration of our solutions. I just received some more samples of the High Purity TTX for double checking the earlier findings.

11-oxo TTX. This compound is a natural analogue discovered in a southern species of Pacific puffer, Arothron nigropuntatus, and proven structurally to be a hydrated aldehyde of TTX (4). We did not receive any samples for assay. However, we have produced this compound in the lab by oxidation of TTX in our chemical experiments to produce a specifically labelled TTX (see below). It has about 5 times the potency of TTX in blocking the sodium channel (5). This is the only analogue of TTX known which

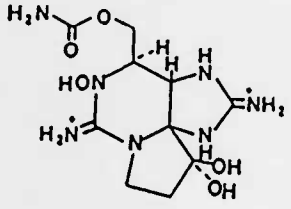
is more potent than TTX itself. We attribute the increased potency to the additional -OH group in the structure, and interpret it to indicate the presence of another hydrogen-acceptor site in the receptor.

Resynthesized TTX - In the chemical work for labelled TTX, 11-oxoTTX is reduced back to TTX. To avoid wasting expensive radioactive chemicals, the reaction was first tried with non-radioactive reagents. The expected TTX in the product was isolated and purified by HPLC (see below). The product TTX is what is termed "resynthesized TTX". On the voltage-clamped skeletal muscle fiber, this product has the identical actions and potency as TTX obtained commercially.

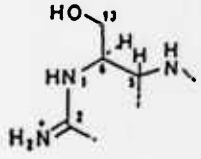
Deoxydecarbamoylsaxitoxin (deoxydcSTX), decarbamoylneosaxitoxin (deneoSTX) and neosaxitoxin (neoSTX). These compounds are natural analogues of STX. I have also reported their actions in the annual report of May 1990, but bring them in here to facilitate the presentation of the TTX/STX binding site. Their structures are shown below.



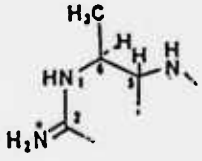
STX



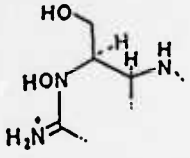
neoSTX



dcSTX



deoxydcSTX



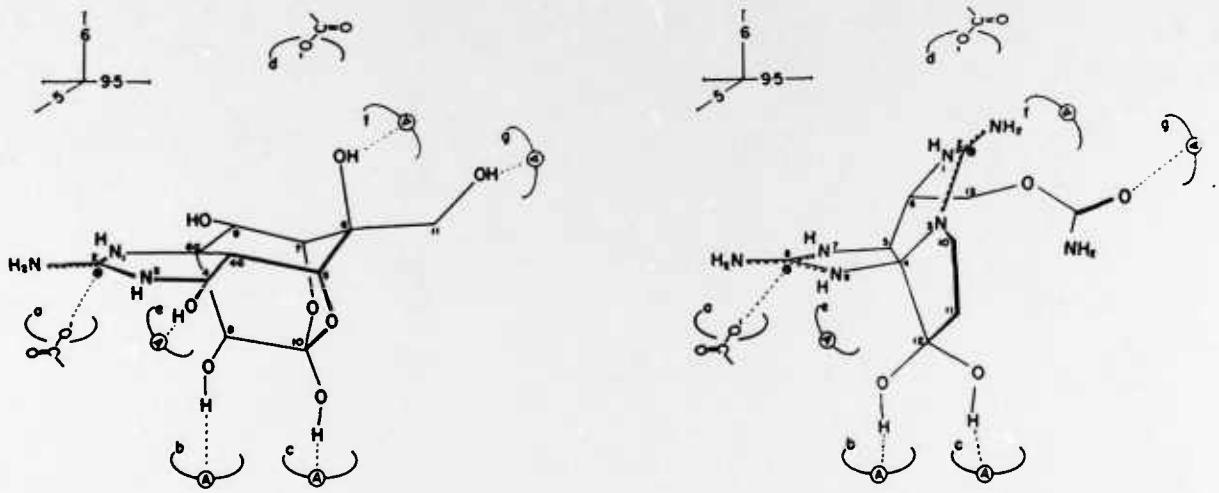
dcneoSTX

Results obtained with these compounds led to conclusions that the receptor has a site-point which has a negative charge (probably a deprotonated carboxylate group) that interacts with the N-1 -OH in neoSTX (6). There are also groups that interact with the C-13 -OH of dcSTX and with the C-14 carbonyl oxygen. The latter two groups turn out to coincide with those that interact with the C-6 -OH and C-11 -OH of TTX.

From all information on structure-activity relations of TTX and STX analogues in hand, we have extended the original 3-group similarities to 6-group similarities:

TTX		STX	
1,2,3	guanidinium	7,8,9	guanidinium
C-9	-OH	C-12	-OH's
C-10	-OH		
C-6	-OH	C-13	-OH (in dcSTX)
C-11	-OH	C-14	carbonyl
----		N-1	-OH (in neoSTX)
C-4	-OH	-----	
C-8	-OH	-----	

Probable TTX/STX binding site. I have also reported on our preliminary thinking on this binding site in the annual report of May 1, 1990. After much further consideration and some molecular modelling, we confirm those early thinking. Two views of the TTX and STX molecules and the same anchoring site-points are shown below.



The left panel is for TTX, and the right for STX. In these views, the anchoring site-points a - g are identical. Site a is most likely a carboxylate function with a anionic charge at body pH. This point forms an ion-pair with the cationic active guanidinium group in the TTX and STX molecules. Sites b and c form hydrogen-bonds with the C-9 and C-10 -OH's of TTX or the gem-diols of STX. Site d is probably a carboxylate which interacts with the N-1 -OH of neoSTX, but may have some ion-pair effect on the 1,2,3 guanidinium of STX and neoSTX. Site e forms a hydrogen-bond with the C-4 -OH of TTX, and may not be involved in binding STX. Site f forms a hydrogen-bond with C-6 -OH of TTX, and the C-13 -OH of decarbamoylSTX, Site g hydrogen-bonds with C-11 -OH of TTX and the carbonyl oxygen of STX. The overall dimensions of the binding site is about 9.5 Å wide, 6 Å tall and 5 Å deep.

In this view, the ChTX molecule is oriented much like the TTX molecule because it contains all the active groups of TTX. However, the large glycine moiety projects out of the plane of the paper in the direction of the entrance to the binding pocket. In this orientation, the reactive groups on glycine do not interact with any residues in the receptor. This view of the the TTX/STX binding site also explains two features about some STX analogues which have never been satisfactorily explained. In this view, C-11 sulfated gonyautoxins will have their sulfate groups project into a region where not much interaction could occur. Such an orientation explains the relatively minor effects the large and strongly negative sulfate groups have on potency. On the other hand, when the same sulfate groups are attached to the end of the carbamoyl side-chain of STX, then it causes steric hinderance, preventing the toxin molecule from properly occupying the receptor site.

Recently, a single-point mutation of glutamate 387 of rat brain sodium channel II to glutamine reduced the TTX and STX binding by 3 orders of magnitude (7). If we accept that observation and interpret it to mean that glutamate 387 with its anionic charge is equivalent to our site a, then molecular modelling shows that asparagine 388 provides sites b and c by way of its carbonyl oxygen in its carbamoyl end. In rat cardiac muscle sodium channel, residue 388 is arginine, and this sodium channel is relatively insensitive to TTX or STX, with a K_d of micromol as contrasted with the K_d of nanomols of sensitive sodium channels (8). The TTX/STX insensitivity could be due to a neutralization of the anionic charge of glutamate 387 by the cationic guanidino function of arginine (7). On the basis of molecular modelling, I believe that as important as charge neutralization may play, the loss of the hydrogen-bonding sites b and c is also important.

Identification of other sites with specific amino acid residues has not been successful so far, because the sequence data now available provide only a linear order of amino acid residues, whereas the TTX/STX binding site is enveloped on 3 sides, undoubtedly with participation of residues far removed from the 387 and 388 loci. The only way to identify the amino acid residues would be to isolate the binding site by use of marker substances, and then conducting peptide-mapping of the labelled residues.

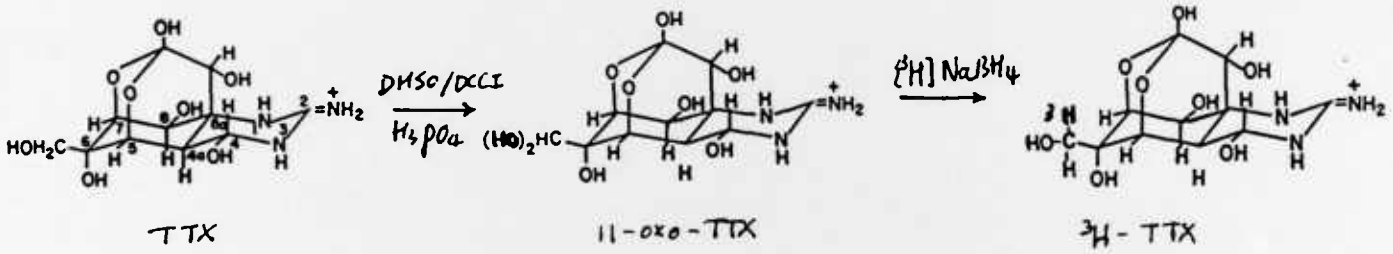
Chemical Work

Tritium-labelled TTX. The only marker compounds available for the TTX/STX binding site are radioactively labelled TTX and STX. Of the two, the latter is the more commonly used. It is made by exchange diffusion of tritium onto the C-11 methylene hydrogen at ca. 45°C, and then stored at low temperature to reduce loss of radioactivity through back diffusion and exchange into aqueous solvent. It has a specific activity of about 0.5 Ci/mol. The available radioactive TTX is made by exposing TTX to an electric arc while in a vapor of tritium water (Wilzbach method). The yield is about 1%, and because radioactivity is also present on unknown degraded material, the product requires extensive cleaning-up work. The TTX molecule is diffusely labelled with a specific activity also around 0.5 Ci/mol. Another class of radioactive TTX comes in the form of TTX coupled (via ethylene diamine at the C-6 position) to some radioactive amino acids.

None of these compounds are readily available. Although they have been used efficiently to isolated the sodium channel, they are unsuitable for more detailed studies of the peptide structure of the binding site for at least two reasons:

(a) The ethylene diamine coupled TTX derivatives are too large to be binding unequivocally only to the TTX/STX binding site. In view of our proposal above of the probable size and shape of that binding site, it is doubtful that the label could be on tne binding site. (b) The specific activities of the tritium-labelled toxins are too low, and they also suffer from exchange diffusion with solvent protons during the experiment. Another deficiency concerns the reliability of the character of the product, such as the ethylene diamine coupled derivatives. The first step used in the synthesis of those compounds was an oxidation of TTX to a reactive derivative, which was then used for further synthesis. However, the nature of the oxidation product is completely unknown. Nothing was ever isolated or characterized; all claims were based on theorectical expectations of anticipated reactions.

In the past year, we have made a specifically labelled TTX of high specific activity. The basis of the reaction is:



Oxidation of TTX to 11-oxo TTX is accomplished by use of the Pfitzner-Moffit method. Although this reaction has been used before by Chicherportiche et al. (8) in their synthesis of the ethylene diamine coupled amino acid derivatives of TTX, their reaction product from this oxidation appears to have been nortetrodotoxin rather than 11-oxo TTX. Indeed, at the time of that work, 11-oxo TTX was not a known compound: an aldehyde of TTX had been sought in some earlier chemical structural studies, but without success. The oxidation product then known was norTTX, a ketone which actually existed in an uncertain equilibrium of several states.

When we first successfully made 11-oxoTTX synthetically in the laboratory, we were unaware of any other such work. Later, we learned that our Japanese colleagues have also succeeded. We then collaborated closely with them to refine the procedures. We could only do this work after Yasumoto and his colleagues first isolated 11-oxoTTX as a natural analogue, and proven its structure. We also needed to have the HPLC-based TTX-analyzer to separate 11-oxoTTX from TTX and other contaminants. We have now characterized our product (a) chromatographically by comparison with an authentic sample of 11-oxoTTX, and (b) by NMR spectrum.

The 11-oxoTTX was then reduced back to TTX. Both the 11-oxoTTX and the resynthesized TTX were tested for sodium-channel blocking activity (see above). After demonstrating that the reaction scheme is feasible, and that the product has the identical biological activity as TTX, we used sodium borotritide as the reagent for reducing 11-oxoTTX. The product is a ^{3}HTTX, specifically labelled on one of the methylene hydrogens. The yield of the oxidation step is about 15%, and that for the reduction step is about 40%. So the overall yield is about 6%. This yield is lower than we expected because of the unexpectedly low yield in the reduction step. The reasons for this low yield are not entirely known, but we could not find ways to improve the yield at this time because of the limited amount of 11-oxoTTX on hand.

Nevertheless, the overall yield is still 6 times better than the Wilzbach method of producing diffusely labelled TTX. Our product needs no clean-up work.

Our ^{3}HTTX has a specific activity of ca. 3000 Ci/mol, or nearly 6,000 times better than the labelled STX or labelled TTX currently available. Another important advantage of this new labelled product is its stability, because the tritium label is in a position where it does not exchange with solvent protons. It is now being evaluated by Dr. S. R. Levinson for specific binding properties to isolated sodium channels.

Our aims with regard to this labelled TTX are: (a) to improve the overall yield, and (b) to use it in the next project of directly marking the TTX/STX binding site for peptide-mapping studies. The methods developed in this work for making the ^{3}HTTX are also the subject of a patent application.

Synthetic agonists and antagonists of TTX. The TTX molecule contains 5 rings. Previosuly we have tried numerous attempts to make some synthetic compounds with some resemblemce to TTX. All those efforts were centered around cyclic guanidinium compounds with two proximate hydroxyl groups. Those efforts were based on our identification of a guanidinium function and two neighboring hydroxyl groups as being critical for sodium-channel blockade. However, such compounds are intrinsically unstable, because of a tendency for intramolecular rearrangements.

Because of our new and expanded view of the TTX/STX binding site (see above) additional anchoring site-points have been identified. Such a recognition broadens the possibility of synthesizing other compounds which can have some mimietic or antagonistic effects to TTX and STX. These efforts are still at an early stage, and no compound has yet been put "into a bottle" for testing purposes. I will continue to report our progress in a timely manner on these developments.

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INFORMATION

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012

REPLY TO
ATTENTION OF:

AD-B156 671

SGRD-RMI-S (70-1y) **ERRATA** 7 APR 1994

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Building 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research, Development, Acquisition and Logistics Command (USAMRDALC)(Provisional), has reexamined the need for the limited distribution statement on the technical reports for Contract No. DAMD17-87-C-7094. Request the limited distribution statement for ADB124671, ADB134376, ADB145447, and ADB156671 be changed to "Approved for public release; distribution unlimited." Copies of these reports should be released to the National Technical Information Service.

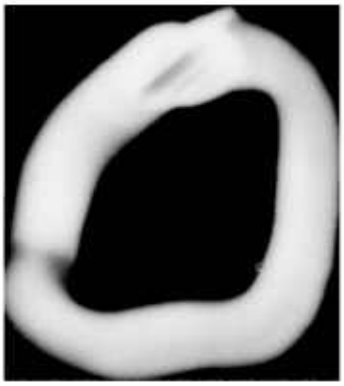
2. Point of contact for this request is Mrs. Judy Pawlus, DSN 343-7322.

151, 20/14

AD-B156 671 **ERRATA**

FOR CAREY O. LEVERETT
LTC, MS
Deputy Chief of Staff for
Information Management

M/O 10 May 94, Done



SUPPLEMENTARY

INFORMATION

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012

REPLY TO
ATTENTION OF:

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ERRATA

7 APR 1994

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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research, Development, Acquisition and Logistics Command (USAMRDALC)(Provisional), has reexamined the need for the limited distribution statement on the technical reports for Contract No. DAMD17-87-C-7094. Request the limited distribution statement for [redacted], and ADB156671 be changed to "Approved for public release; distribution unlimited." Copies of these reports should be released to the National Technical Information Service.

2. Point of contact for this request is Mrs. Judy Pawlus, DSN 343-7322.

151, 20/14

ERRATA -AD-B156671/

Pawlus Pinehart
for CAREY O. LEVERETT
LTC, MS
Deputy Chief of Staff for
Information Management

A/O 10 May 94, Done