



National Library
of Canada

Bibliothèque nationale
du Canada

Acquisitions and
Bibliographic Services Branch

Direction des acquisitions et
des services bibliographiques

395 Wellington Street
Ottawa, Ontario
K1A 0N4

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file *Votre référence*

Our file *Notre référence*

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

**THE PURIFICATION, CHARACTERIZATION AND MODE
OF ACTION OF *BACILLUS THURINGIENSIS* SUBSPECIES
ISRAELENIS PROTEINS**

Fang Huang

A thesis submitted to the School of Graduate Studies and Research of the
University of Ottawa in partial fulfilment of the requirement for the degree of
Master of Sciences

Department of Biochemistry
Faculty of Medicine
University of Ottawa
Ottawa, Canada

© Fang Huang, Ottawa, Canada. 1994



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file *Votre référence*

Our file *Notre référence*

THE AUTHOR HAS GRANTED AN
IRREVOCABLE NON-EXCLUSIVE
LICENCE ALLOWING THE NATIONAL
LIBRARY OF CANADA TO
REPRODUCE, LOAN, DISTRIBUTE OR
SELL COPIES OF HIS/HER THESIS BY
ANY MEANS AND IN ANY FORM OR
FORMAT, MAKING THIS THESIS
AVAILABLE TO INTERESTED
PERSONS.

L'AUTEUR A ACCORDE UNE LICENCE
IRREVOCABLE ET NON EXCLUSIVE
PERMETTANT A LA BIBLIOTHEQUE
NATIONALE DU CANADA DE
REPRODUIRE, PRETER, DISTRIBUER
OU VENDRE DES COPIES DE SA
THESE DE QUELQUE MANIERE ET
SOUS QUELQUE FORME QUE CE SOIT
POUR METTRE DES EXEMPLAIRES DE
CETTE THESE A LA DISPOSITION DES
PERSONNE INTERESSEES.

THE AUTHOR RETAINS OWNERSHIP
OF THE COPYRIGHT IN HIS/HER
THESIS. NEITHER THE THESIS NOR
SUBSTANTIAL EXTRACTS FROM IT
MAY BE PRINTED OR OTHERWISE
REPRODUCED WITHOUT HIS/HER
PERMISSION.

L'AUTEUR CONSERVE LA PROPRIETE
DU DROIT D'AUTEUR QUI PROTEGE
SA THESE. NI LA THESE NI DES
EXTRAITS SUBSTANTIELS DE CELLE-
CI NE DOIVENT ETRE IMPRIMES OU
AUTREMENT REPRODUITS SANS SON
AUTORISATION.

ISBN 0-612-00584-4

Canada



UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

Abstract

The gram-positive, spore forming bacteria *Bacillus thuringiensis* synthesize cytoplasmic crystalline inclusions, the Bt proteins, which are toxic to insect larvae. Although Bt proteins have been used as bioinsecticides for many years and a considerable number of studies have been carried out to investigate their mode of action, their precise mechanism of action still remains unclear.

A 27 kDa protein from Bt subsp. *israelensis* (cytA27) is known for its broad cytolytic activity against a wide range of invertebrate and vertebrate cells *in vitro* as well as mosquitocidal activity *in vivo*. Neither its three-dimensional structure nor its mode of action are known. Previous studies have suggested that the cytA27 protein interacts with certain phospholipids, making phospholipid vesicles permeable without a requirement for a specific receptor. These results as well as the unique broad cytolytic feature of the cytA27 protein indicated that the mechanism of action of this protein may not resemble the putative mode of action for the main family of B.t. proteins. Given the interest in the mechanism of the membrane interaction of cytA27 and its proteolytic product, a 24 kDa protein (cytA24), we have undertaken a study of their interactions with model phospholipid membranes. The cytA27 and cytA24 proteins were purified by

selective solubilization and ion exchange HPLC and characterized by SDS-PAGE, N-terminal sequencing and mass spectrometry. Fluorescence spectroscopy was used to characterize the cytA-induced release of fluorescence markers from vesicles and the cytA-vesicle affinity and stoichiometry.

The results indicate that cytA proteins bind to lipids and affect the integrity of phospholipid vesicles. The binding is non-specific in that it does not require a receptor. CytA27 and cy8tA24 interact with PC-LUV with apparent binding constants of $(0.34 \pm 0.02) \times 10^5 \text{M}^{-1}$ and $(1.54 \pm 0.10) \times 10^5 \text{M}^{-1}$, respectively. Binding isotherm data indicate that a critical number of cytA molecules must associate with the membrane in order to induce vesicle leakage. Approximately 324 of cytA27 and 157 of cytA24 molecules are required to bind to one PC-LUV before the latter starts to release its contents. CytA-induced vesicle leakage follows an all-or-none mechanism, i.e. each LUV either releases all of its contents or remains intact. Experiments on the release of fluorescence markers from vesicles by cytA proteins demonstrate that both small (calcein) and large (3,000 and 10,000 Dalton dextran) molecules are released from phospholipid vesicles with similar kinetics. These results suggest that the cytA proteins do not create small, selective proteinaceous channels but rather cause non-specific membrane perturbations.

ACKNOWLEDGEMENTS

I would like to express my grateful thanks to Dr. P. Carey and Dr. M. Pusztai-Carey for giving me the opportunity to undertake graduate studies. Without their guidance, patience, encouragement throughout my study, this work would not have been possible. I would like to especially thank them for being supportive during the difficulties in the research environment that happened in the past year.

All the fluorescence spectroscopy experiments were carried out in cooperation with Dr. P. Butko and Dr. W. Surewicz. They actively participated in all aspects of this work. I sincerely appreciate the numerous contributions they made toward this thesis. A special thanks to Dr. Butko for being there to answer all the questions, his endless patience, and sharing his extensive knowledge in this field and friendship.

I would also like to express many thanks to Mr. D. Watson and Dr. M. Yaguchi for performing amino acid sequencing and mass spectra analysis, allowing me to learn the techniques in their laboratory and providing invaluable advice.

I thank all members of Dr. M. Pusztai-Carey's group for their contribution. Especially, I would like to thank Mr. H. van Faassen for his technical assistance, suggestion and for providing a good working environment.

Thanks to Dr. P. Anderson, Dr. W. Surewicz and Dr. G. Willick for participating in my advisory committee and for giving helpful suggestions and criticisms regarding this work.

I am grateful to all my fellow graduate students at Institute for Biological Sciences, NRC, T. Dahms, J. Doran and C. Hogue for moral support and friendship.

I acknowledge the financial support provided by the Abbott Company (Chicago, IN, USA) for this research.

I dedicate this thesis to my family and to the memory of my mother.

Table of Contents

Abstract	i
Acknowledgements	iii
List of figures	vii
List of tables	x
List of Abbreviations	xi
Introduction	
1 Overview	1
2 Classification of <i>Bacillus thuringiensis</i> proteins	2
3 History	5
4 Molecular genetics of Bt and transgenic plants	7
5 The mode of action and the structure of Bt proteins	12
6 The <i>B. thuringiensis</i> protein possessing broad cytolytic activity	17
7 Objective of the present study	23
Materials and methods	
1 Materials	25
2 <i>Bacillus thuringiensis</i> subsp. <i>israelensis</i> crystal isolation	25
3 Isolation of the cytA27 and cytA24 cytolytic proteins from <i>Bacillus thuringiensis</i> subsp. <i>israelensis</i> crystals	27
4 N-terminal amino acid sequencing	29
5 Mass spectral analysis	29
6 Haemolysis experiment	30
7 Fluorescence assay of cytA protein-induced permeabilization of phospholipid vesicles	31
8 Release mechanism: 'all or none' or 'graded release'	34
9 Tryptophan fluorescence quenching by acrylamide or iodide	36

Results

1	<i>Bacillus thuringiensis</i> protein crystal preparation	38
2	The cytA27 and cytA24 protein purification and characterization	38
3	Haemolysis experiments	51
4	CytA27 and cytA24 induced release of fluorescence markers from lipid vesicles	51
5	The mechanism of the cytA proteins induced calcein release	61
6	Determination of cytA protein-lipid binding isotherms	68
7	Quenching of the tryptophan fluorescence of the cytA27 and cytA24 proteins	76
	Discussion	84
	References	95

List of Figures

Figure 1	SDS - PAGE gel of solubilization of <i>Bacillus thuringiensis</i> subsp. <i>israelensis</i> proteins in different buffers	42
Figure 2	Purification of the cytA27 protein from solubilized <i>B. thuringiensis</i> subsp. <i>israelensis</i> crystal proteins using ion-exchange HPLC	43
Figure 3	SDS-PAGE of the cytA27 protein and cytA24 protein separation	44
Figure 4	Chromatogram of the cytA24 protein isolation by ion-exchange HPLC	45
Figure 5	Elution profile of trypsin on a Protein Pak SP 5PW column and chromatogram of the cytA24 protein isolation by ion-exchange HPLC	46
Figure 6	The predicted amino acid sequence and calculated molecular weight of the cytA27 protein	47
Figure 7	The predicted amino acid sequence and the calculated molecular weight of trypsin digestion product of the cytA27 protein	48
Figure 8	Electrospray mass spectrum and report of the cytA27 protein	49
Figure 9	Electrospray mass spectrum and report of the cytA24 protein	50
Figure 10	Haemolytic activities of the cytA24 and cytA27 proteins	52
Figure 11	Typical time course curves of calcein leakage from egg PC vesicles induced by cytA proteins.	54

Figure 12	Kinetics of the cytA27 protein induced calcein release from PC-LUV at pH 4.0	55
Figure 13	Kinetics of the cytA27 protein induced calcein release from PC-LUV at pH 7.4	56
Figure 14	Kinetics of the cytA27 protein induced calcein release from PC-LUV at pH 10.0	57
Figure 15	Effect of pH on cytA27 induced leakage of entrapped calcein from PC-LUV	58
Figure 16	Kinetics of the cytA24 protein induced calcein release from PC-LUV at pH 7.4	59
Figure 17	The extent of release of the cytA24 and cytA27 proteins induced calcein leakage from PC-LUV vesicles at a variety of protein and lipid ratios	62
Figure 18	The extent of release of the cytA27 and cytA24 proteins induced calcein leakage from PC-SUV vesicles at a variety of protein and lipid ratios	63
Figure 19	The cytA24 protein induced FITC-dextran release from PC-LUV	64
Figure 20	Experiments on the mechanism of the cytA proteins induced vesicle leakage	66
Figure 21	The standard quench curve of the fluorescence quenching as a function of the entrapped calcein concentration in LUV	67
Figure 22	Dependency of calcein leakage rates on the cytA27 protein concentration at three different lipid concentrations	71

Figure 23	Leakage rate of calcein release as a function of the cytA24 protein concentration at four different lipid concentrations.	72
Figure 24	Estimation of free and membrane-bound protein concentrations at several leakage rates	73
Figure 25	Quantitative binding isotherm for the interaction of the cytA proteins with PC-LUV vesicles	74
Figure 26	Dose response curves relating leakage rate to the molar ratio r of bound cytA to lipid	75
Figure 27	Quenching of the cytA27 protein tryptophan fluorescence by acrylamide in the absence and presence of egg PC-SUV	79
Figure 28	Quenching of the cytA24 protein tryptophan fluorescence by acrylamide in the presence and absence of egg PC-SUV	80
Figure 29	Quenching of the cytA27 protein tryptophan fluorescence by potassium iodide in the absence and presence of egg PC-SUV	81
Figure 30	Quenching of the cytA24 protein tryptophan fluorescence by potassium iodide in the absence and presence of egg PC-SUV	82

List of Tables

Table 1	<i>Bacillus thuringiensis</i> subspecies	4
Table 2	Insecticidal crystal protein genes of <i>B. thuringiensis</i>	10
Table 3	Rate constants of cytA27 and cytA24 induced calcein release as a function of protein concentration and pH	60
Table 4	Parameters of the cytA27 and cytA24 protein tryptophan fluorescence quenching by acrylamide (AA) and potassium iodide (KI)	83

List of abbreviations

AA	Acrylamide
BRI	Biotechnology Research Institute
Bt	<i>Bacillus thuringiensis</i>
CAPS	3-(cyclohexylamino)-1-propane-sulphonic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein Isothiocyanate
HEPES	N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulphonic acid)
HPLC	high performance liquid chromatography
IBS	Institute for Biological Sciences
kDa	kilodalton
LUV	Large unilamellar vesicles
NRC	National Research Council
PC	L- α -phosphatidylcholine
SUV	Small unilamellar vesicles
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Introduction

1. Overview:

Formulations of *Bacillus thuringiensis* (generally known as 'Bt') have been used for more than three decades as biological insecticides for agricultural and forestry purposes. Interest in *Bacillus thuringiensis* proteins has increased in recent years, because of the need of an alternative to chemical insecticides which have problems associated with environmental contamination and the development of insect resistance.

Bacillus thuringiensis is a naturally occurring, gram-positive, spore-forming, aerobic bacterium. It is characterized by its ability to synthesize crystalline inclusion bodies during sporulation. These crystalline bodies consist of protein subunits called δ -endotoxins for their parasporal, crystal associated features which differ from other Bt endotoxins or exotoxins. The proteins exhibit insecticidal activity against the larvae of a wide range of agricultural pests and disease-carrying insects. However, they are harmless to human beings, birds, vertebrates, as well as to beneficial insects and to plants. Bt proteins are highly specific in their insecticidal activity with different strains of the bacillus exhibiting different insect host spectra. By 1992, about 36 *Bacillus thuringiensis* subspecies had been identified (Beegle and Yamamoto, 1992), some of which

show a high degree of homology and some of which have very diverse nucleotide and therefore amino acid sequences. Moreover the discovery of further subspecies is expected. The discovery that the genes encoding the crystal proteins are located on plasmids and the successful efforts in cloning these genes have opened the door to the widespread use of molecular genetics in Bt research. The existence of a large number of Bt endotoxins has been one of the proven advantages of its use over most synthetic chemical insecticides. Since Bt crystals often contain more than one kind of endotoxin, the chances of resistance developing to all the δ -endotoxins simultaneously should be reduced, therefore preventing or at least postponing the development of insect resistance. Another favourable aspect of Bt proteins is that they are readily degraded in the environment. Taken together, these features make Bt proteins highly desirable for pest control.

As a consequence of the reduced cost of Bt products and of the environmental imperatives, Bt sales are rapidly expanding and now represent 90-95% of the total biopesticide market. This market has grown from \$24M in 1980 to \$107M in 1989, and is predicted to expand at an annual rate of 11% to reach \$300M by 1999 (Feitelson *et al.*, 1992).

2. Classification of *Bacillus thuringiensis* proteins:

Several methods have been used to classify *B. thuringiensis* strains into serotypes, subspecies (subsp.) or varieties (var.). The most commonly used classification system is based on their flagellar antigens. By 1992, Bt strains have been classified into about 36 subspecies based on their serotype and some biochemical and host range information and are listed in Table 1 (Beegle and Yamamoto, 1992). However, this nomenclature system fails to reflect the structure or large diversity in insect specificity of the inclusion proteins in a consistent manner. Recently, a new classification system has been developed by Whiteley and co-workers. The crystal proteins and their genes have been classified based on their structure, antigenic properties and insecticidal activity into four major groups: cryI (lepidoptera-specific), cryII (lepidoptera and diptera-specific), cryIII (coleoptera-specific) and cryIV (diptera-specific) (Höfte and Whiteley, 1989; Adang, 1991). In addition, there is an apparently unique cytolytic Bt protein with broad cytolytic activity, cytA, as well as a newly discovered toxin designated cryV which is active against coleoptera and lepidoptera (Tailor *et al.*, 1992).

Table 1. *Bacillus thuringiensis* subspecies (Beegle and Yamamoto, 1992)

H-serotype	Subspecies
1	<i>thuringiensis</i>
2	<i>finitimus</i>
3a	<i>alesti</i>
3a, 3b	<i>kurstaki</i>
3a, 3d	<i>sumiyoshiensis</i>
3a, 3d, 3e	<i>fukuokaensis</i>
4a, 4b	<i>sotto</i>
4a, 4b	<i>dendrolimus</i>
4a, 4c	<i>kenyae</i>
5a, 5b	<i>galleriae</i>
5a, 5c	<i>canadensis</i>
6	<i>subtoxicus</i>
6	<i>entomocidus</i>
6a, 6c	<i>oyamensis</i>
7	<i>aizawai</i>
8a, 8b	<i>morrisoni</i>
8a, 8b	<i>tenebrionis</i>
8a, 8c	<i>ostrinae</i>
8b, 8d	<i>nigeriensis</i>
9	<i>tolworthi</i>
10	<i>darmstadiensis</i>
11a, 11b	<i>toumanoffi</i>
11a, 11c	<i>kyushuensis</i>
12	<i>thompsoni</i>
13	<i>pakistani</i>
14	<i>israelensis</i>
15	<i>dakota</i>
16	<i>indiana</i>
17	<i>tohokuensis</i>
18	<i>kumamotoensis</i>
19	<i>tochiensis</i>
20	<i>yunnanensis</i>
20	<i>pondicheriensis</i>
21	<i>colmeri</i>
22	<i>shandogensis</i>
23	<i>japonensis</i>
—*	<i>wuhanensis</i>

* No flagella

3. History:

Bacillus thuringiensis first claimed the attention of scientists 90 years ago, when a *B. thuringiensis* strain was isolated from diseased silkworm *Bombyx mori* by a Japanese bacteriologist S. Ishiwata (Ishiwata, 1901). He named it Sottokin which translates into "sudden death bacillus" and described the pathology which follows the bacterium's ingestion by silkworm larvae (Ishiwata, 1905). A decade later, in 1911, a similar organism was isolated by Berliner from the larvae of *Anagasta kuehniella* (flour moth) in Germany (Berliner, 1911). Berliner gave the bacterium the formal name *Bacillus thuringiensis*. The first record of using Bt for insect control is against the European corn borer (*Ostrinia nubilalis*) in Hungary by Husz in the late 1920s and early 1930s as part of an international project funded by the U.S.A. (Briggs, 1986). The first commercial product Sporeine became available in 1938 in France (Weiser, 1986). In the early 1950s, work by Steinhaus at the University of California at Berkeley (Steinhaus, 1951; 1956) stimulated interest in the commercialization of Bt proteins in the United States. By 1957, a product called Thuricide was produced by Pacific Yeast Products (later Bioferm Corporation). Landmark studies were undertaken in 1953 and 1954 by Canadian microbiologists Hannay and Angus who proved that the parasporal inclusion bodies were responsible for

the toxicity of *B. thuringiensis* (Hannay, 1953; Angus, 1954). Subsequently, Angus went on to show that the crystalline inclusion bodies are made up in large part of proteins (Angus, 1956).

In the 1960s, a new Bt subspecies *kurstaki* was found based on its flagellar serotype (de Barjac and Lemille, 1970). One of five new Bt subsp. *kurstaki* isolates, HD-1, was isolated from diseased *Pectinophora gossypiella* (pink bollworm) larvae by Dulmage in 1970 (Dulmage, 1970). HD-1 based Bt products proved to be much more effective than the existing products based on *B. thuringiensis* subsp. *thuringiensis*. The discovery of HD-1 significantly improved the effectiveness of Bt in the field and promoted commercialization. In the meantime, the first international standard for evaluating the potency of Bt commercial products was established in 1966 (Burgess, 1966; 1967). A preparation of *B. thuringiensis* subsp. *thuringiensis* E-61, which was produced by Institut Pasteur in Paris, was recommended for adoption as the international primary standard for the bioassay of *B. thuringiensis* preparations. The standardization procedure is based on insect bioassays and compares the resulting LC_{50} s (50% effective dosage) with the LC_{50} of a concurrently bioassayed standard. The standard is assigned a potency of 1,000 International Units (IU) per milligram. The North American standards HD-1-S-1971 and

presently HD-1-S-1980 (Dulmage *et al.*, 1971; Beegle *et al.*, 1986) are now commonly used. Worldwide use of Bt commercial products started in the early 1980s. In North America, forest pest control is by far the most important Bt market, and Bt has started to replace conventional insecticides. This market accounts for about 60% of world-wide sales in the mid-1980s (Burgess and Daoust, 1986).

4. Molecular genetics of Bt and transgenic plants

It has been demonstrated that there are a number of different plasmids present in each Bt cell; the exact number and size of these plasmids vary between subspecies. The plasmids are divided into two groups according to size, small plasmids (<10 megadaltons) and large plasmids (40-150 megadaltons) (Lereclus *et al.*, 1982). Genes encoding Bt crystal proteins are usually found on large plasmids. The possibility that *cry* genes are also located on the chromosome cannot be ruled out. Zakharyan *et al.* (1976) were the first to report the presence of plasmids in *B. thuringiensis* and the pace of research accelerated after Gonzalez and co-workers found that the genes encoding the crystal proteins are located on plasmids which can be transferred between strains by means of a conjugation-like transfer (Gonzalez *et al.*, 1981; 1982). In 1981, Schnepf and

Whiteley reported the cloning of one of the Bt subsp. *kurstaki* crystal protein genes into the pES plasmid in *Escherichia coli*. This triggered numerous reports on the cloning of Bt crystal genes from other subspecies (e.g. Kleir *et al.*, 1982; McLinden *et al.*, 1985; Whiteley *et al.*, 1986). Several gene sequences for the crystal proteins were published in 1985 (Adang *et al.*, 1985; McLinden *et al.*, 1985; Schnepf *et al.*, 1985). The sequence information permitted the construction of specific gene probes for screening strains using hybridization analysis and greatly facilitated the discovery of new Bt genes. The narrow spectrum of insecticidal activity has been a factor limiting the widespread application of *B. thuringiensis* proteins. The use of molecular genetics to manipulate Bt genes facilitates the creation of new strains which have a broader host range. The activity spectrum can be expanded by introducing additional crystal protein genes either by the conjugation of plasmids from other *B. thuringiensis* strains or through direct transformation of crystal protein genes cloned in the *B. thuringiensis* replicon. One successful example of constructing a strain with broader and improved insecticidal activity is the product "Condor" marketed by Ecogen in 1988, which contains both lepidopteran and coleopteran Bt genes combined through a plasmid recombination (Carlton, 1988). There are also several other reports of constructs with broader insecticidal activity spectra

achieved using recombinant DNA technology (Honee *et al.*, 1990; Bassand *et al.*, 1989).

The availability of cloned Bt genes has also led to the bioassay of individual crystal proteins, thus permitting the analysis of each gene product's spectrum of insecticidal activity without interference from other proteins. By 1989 the nucleotide sequences of 42 *B. thuringiensis* genes had been reported. Fourteen subclasses of distinct genes based on sequence homology and the insecticidal spectra of the encoded proteins have been postulated and are shown in Table 2 (Höfte and Whiteley, 1989; Lereclus *et al.*, 1993), which represents the up-to-date classification system for *B. thuringiensis* protein genes. New genes encoding proteins with different nucleotide sequences and new insecticidal activities have been found since 1989 including: cryIE isolated from subsp. *kenyae* which is active against larvae of *S. exigua* (Visser *et al.*, 1990; Bossé *et al.*, 1990); cryIF isolated from Bt subsp. *aizawai* which is highly active against lepidoptera *O. nubilalis* and *S. exigua* (Chambers *et al.*, 1991); cryIIIB isolated from Bt subsp. *tolworthi* which is active against Colorado potato beetle (Sick *et al.*, 1990); as well as cryV which has dual activity against both lepidoptera and coleoptera (Tailor *et al.*, 1992).

Another active area in *B. thuringiensis* research is the generation of insect-

Table 2. Insecticidal crystal protein genes of *B. thuringiensis*
(Lereclus et al., 1993)

Gene type	Host range ^a	Predicted mol. mass (kDa)
<i>cryIA(a)</i>	L	133.2
<i>cryIA(b)</i>	L	131.0
<i>cryIA(c)</i>	L/D	130
	L	133.3
<i>cryIB</i>	L	138
<i>cryIC</i>	L	134.8
<i>cryID</i>	L	132.5
<i>cryIE*</i>	L	130
<i>cryIF*</i>	L	133.6
<i>cryIIA</i>	L/D	70.9
<i>cryIIB</i>	L	70.8
<i>cryIIC</i>	L	69.5
<i>cryIIIA</i>	C	73.1
<i>cryIIIB*</i>	C	74.2
<i>cryIVA</i>	D	134.4
<i>cryIVB</i>	D	127.8
<i>cryIVC</i>	D	77.8
<i>cryIVD</i>	D	72.4
<i>cryVA*</i>	L/C	81.2
<i>cytA</i>	?	27.4

^a Specified host ranges: L, lepidoptera; D, diptera; C, coleoptera.

* newly found gene type.

resistant transgenic plants which have the potential of overcoming the problem of the limited stability of *B.thuringiensis* proteins in the field and the delivery of the toxin to the target insects. There have been numerous attempts to express *B. thuringiensis* toxin genes in plants. In 1987 there were three reports of successful expression in tobacco (Adang *et al.*, 1987; Barton *et al.*, 1987; Vaeck *et al.*, 1987) and one in tomato (Fischhoff *et al.*, 1987). The list of Bt transgenic plants now extends to cotton, potato and poplar, and is expected to grow rapidly (Van Frankenhuyzen, 1993). Presently the major problems pertaining to the use of Bt transgenic plants in agricultural production are the low level expression of *B. thuringiensis* proteins in plants and the possible development of insect resistance. By modification of the *B. thuringiensis* coding sequence, 50 to 100 fold increase of toxin protein expression in cotton plants was obtained. Truncated forms of crystal protein genes cryIA(b) and cryIA(c) were expressed in cotton at sufficient levels to effectively control lepidopteran insects. This was achieved by using an improved promoter in *Agrobacterium Ti* transformation vectors and by modification of key regions of the structural gene through site directed mutagenesis without changing the encoded amino acid sequence (Perlak *et al.*, 1990). The first genetically engineered seed for insect-resistant crops is expected to reach the market place within a few years.

5. The mode of action and the structure of Bt proteins

Although proteinaceous parasporal crystals have been observed in *B. thuringiensis* cells since the discovery of the bacillus, their role in pathogenicity was not recognized until the 1950s. Since then, extensive studies have been carried out regarding the molecular biology, protein chemistry, and mode of action of the crystals. The mode of action has been a topic of keen interest as Bt has increased in commercial potential and application in agricultural and forestry pest control. The present understanding of the mode of action of Bt proteins is as follows: *Bacillus thuringiensis* crystalline inclusions may consist of one or several nonidentical insecticidal crystal proteins called protoxins. These are single chain polypeptides with molecular weights ranging from 27 to 140 kDa. In most cases, the parasporal bodies are bipyramidal crystals. They dissolve in the alkaline midgut of the insect and release Bt protoxins; a process which is determined by the insect midgut environment and the composition of the crystals. The solubilized crystal proteins are proteolytically converted into smaller toxin polypeptides which are the actual toxic fragments. The efficiency of this step is determined by the protease composition of the insect midgut and the protein structure. The activated toxin then interacts with the gut epithelium cells; in the case of the cryI proteins the toxin molecules bind to specific

receptors on the brush border membrane of the midgut columnar cells. This may result in the generation of pores in the plasma membrane thereby breaking down the permeability barrier and disturbing the osmotic balance. The cells swell and lyse, with gut integrity being disrupted and with the eventual effect on the insects of causing cessation of feeding and death (Gill *et al.*, 1992; Höfte and Whiteley, 1989; Knowles and Dow, 1993). It is assumed that most Bt proteins, at least in the *cry* family of Bt proteins, behave similarly in the gut of the insect, although this has not been demonstrated directly.

As presented above, the model for Bt's mode of action is a multi-step process, thus the insecticidal activity of Bt proteins could possibly be enhanced by modifying any of the steps involved. Successful interventions have been made at two stages in the mode of action. One example concerns the use of commercial feeding stimulants, as additives to a starch based formulation of Bt (Bartlet *et al.*, 1990). The second focuses on the stabilization of toxin in the insect midgut by the addition of protease inhibitors (MacIntosh *et al.*, 1990). In principle, all of the steps involved in the mode of action should be capable of modification if we can thoroughly understand them, providing impetus for studies on the mode of action at the cellular level.

Manipulation of the genes encoding for Bt proteins and the subsequent

cloning into heterologous hosts greatly facilitate studies on the mode of action. This permits the isolation of individual Bt proteins and individual protein domains from each toxin. It is generally believed that the binding of toxin to the receptor sites existing on the insect midgut cells plays a key role in the determination of insecticidal spectra and toxicity. In several cases, the receptors seem to be glycoproteins (Knowles *et al.*, 1984; Knowles and Ellar, 1986; Haider and Ellar, 1987). The complexity of the situation is illustrated by the fact that a susceptible insect can have binding sites for several different toxins and that a single toxin can recognize more than one binding site. Differences in affinity and concentration of binding sites as well as protoxin stability, differential solubilization of crystals and subsequent proteolytic processing can all contribute to the large diversity of toxicity spectra (Van Frankenhuyzen, 1993; Hofmann *et al.*, 1988; Van Rie *et al.*, 1990).

Numerous studies have been carried out on the relationship between function and structure of Bt proteins. The involvement of protein domains, each exerting a specific function, has been discussed often. Among several cryI crystal proteins, it has been proposed based on sequence analysis that the protoxins have similar conformations and toxins also have similar conformations (Visser *et al.*, 1993). For these molecules, the intact protoxin molecule (130 kDa) consists

of two distinct parts; the N-terminal part of the protoxin, encompassing the toxic fragment, is mainly hydrophobic and shows a lesser degree of homology (40-90%); the C-terminal part of the molecule runs approximately from residue 600 to residue 1150 and shows higher degree of homology (>90%). The trypsin-resistant toxin domain which is located in the N-terminal part of the protoxin is generated by cleavage at approximately amino acid residue 600 and by removal of the 28 amino acids at the N-terminus. The toxin fragment itself appears to consist of two or three independent structural regions often referred to as domains, possibly linked by a protease-sensitive region. The N-terminal portion of the toxic fragments shows a high degree of α -helix structure and of hydrophobicity and may be responsible for cell membrane disruption (either by bringing about pore formation or by disturbing the lipid membrane). The C-terminal portion of the toxin contains mainly β -sheet and coil structure and is considered to be responsible for determining the toxin specificity via differential binding to cell receptors (Convents *et al.*, 1990; 1991; Choma *et al.*, 1990; 1991).

Significant progress was made recently in understanding the relationship between structure and function by David Ellar and colleagues at the University of Cambridge, who published the structure of a coleopteran-specific δ -endotoxin

(cryIII) from Bt subsp. *tenebrionis* based on X-ray crystallographic data at 2.5Å resolution (Li *et al.*, 1991). The X-ray derived structure confirms the multidomain toxin hypothesis. The toxin molecule comprises three domains called domains I, II, and III. Domain I, the so-called toxin domain consists of seven α -helices in an amphipathic bundle, and is likely to be involved in membrane penetration. Domain II is a three β -sheet structure and is believed to be the receptor binding domain. Domain III is a β -sandwich containing the C-terminal region which is conserved in most *cry* proteins, and is presumed to be important in the structural integrity and stability of the toxin. Since the five sequence blocks that are highly conserved throughout the *B. thuringiensis* δ -endotoxin family correspond to the domains which form the core structure of the cryIII toxin molecule, Li *et al.* proposed that their model may also represent the structure of other members of Bt protein family. Based on the above structural knowledge of *cry* toxins, a putative mechanism of pore formation by *cry* toxins has been proposed (Gill *et al.*, 1992). The receptor binding domain (domain II) binds specifically to a receptor on the apical membrane of the columnar cells. Binding triggers a conformation change in the toxin, which induces the toxic domain (pore formation domain, domain I) to penetrate into the cell membrane. Several toxin molecules may aggregate and form a pore, which leads to osmotic

imbalance and cell lysis. Additional knowledge on the structure of Bt proteins and their binding to receptors is needed to further elucidate the relationship of structure and function and to provide the basis for engineering the toxin proteins to achieve greater selectivity and potency.

6. The *B. thuringiensis* toxin possessing broad cytolytic activity

Intact parasporal crystals produced by the *B. thuringiensis* subspecies *israelensis* are toxic to the larvae of several dipteran insects including mosquitos and black flies. *Israelensis* was the first isolate of *B. thuringiensis* to show mosquitocidal activity, although a number of *B. thuringiensis* subspecies effective against mosquitos have been found subsequently. The common feature of this group of Bt proteins is the complex polypeptide profiles of their parasporal inclusions. For example, the parasporal crystals of Bt subspecies *israelensis* contain at least four mosquitocidal proteins with molecular weights 135, 128, 65, 27 kDa (Chilcott and Ellar, 1988); *B. thuringiensis* subsp. *darmstadtensis* 73-E10-2 synthesizes crystal proteins of 125, 83, 79, 77, 69, 50 and 27 kDa (Drobniewski and Ellar, 1989); and Bt subspecies *fukuokaensis* produces crystal proteins of 90, 86, 82, 72, 50, 48, 37, and 27 kDa (Yu *et al.*, 1991). Each of these proteins is a protoxin which can be converted into a

smaller toxic polypeptide by proteases.

Typically, the parasporal body of Bt subsp. *israelensis* is spherical in shape and averages about 1 μ m in diameter. It has been found that the parasporal body consists of at least three different types of protein inclusions which are bound together by a laminated netlike envelope of undetermined composition. The protein inclusions differ from one another in size, shape, electron density and lattice spacing. The largest inclusion makes up 40-50% of the parasporal body and is characterized as being rounded to polyhedral. It is thought to contain the 27 kDa protein. The second type of inclusion appears bar-shaped and makes up 15-20% of the parasporal body and consists almost exclusively of a 65 kDa protein. The third inclusion type is hemispherical to spherical, constitutes 20-25% of the parasporal body and is thought to contain the proteins of 128 and 135 kDa molecular weight (Federici et al., 1990)

Among the four major proteins contained in the intact parasporal body of Bt subsp. *israelensis*, the 27 kDa protein has received the most attention. The 27 kDa protein from *B. thuringiensis* subsp. *israelensis* is referred to here as cytA27 protein and its proteolytic cleavage product, a 24 kDa protein, is referred to as cytA24 protein. The cytA proteins differ markedly in size and their amino acid sequences from the known proteins commonly found in other

Bt subspecies, e.g. in the Bt strains effective against lepidoptera the protoxins have molecular weight about 130 kDa and the corresponding toxins are about 65 kDa. The cytA27 protein is known for its broad cytolytic and haemolytic activities *in vitro* as well as its mosquitocidal activity *in vivo* (Thomas and Ellar, 1983a; Armstrong *et al.*, 1985; Chilcott and Ellar, 1988) and studies have even suggested that this endotoxin may act as a neurotoxin (Cheung *et al.*, 1985; Chilcott *et al.*, 1984). Thomas and Ellar (1983a) were the first to detect the broad cytolytic activity of cytA27 protein against a wide range of invertebrate and vertebrate cells *in vitro*. Other biochemical and cloning experiments (Hurley *et al.*, 1985; Bourgouin *et al.*, 1986; Held *et al.*, 1986) have provided confirmation that the cytA27 protein is responsible for the haemolytic and cytolytic activities. Thomas and Ellar and other authors (Armstrong *et al.*, 1985; Davidson and Yamamoto, 1984) attributed the mosquitocidal toxicity of *Bt israelensis* to the cytA27 protein. However, other authors (Hurley *et al.*, 1985; Lee *et al.*, 1985) have proposed that the 65 kDa or the 130 kDa protein is the actual mosquitocidal toxin. More recent studies by Wu and Chang (1985) and Chilcott and Ellar (1988) have shown that the cytA27 protein is indeed mosquitocidal, but it is not toxic enough to account for the total toxicity of the intact parasporal body. It has been suggested that the high toxicity of the

parasporal body is not due to a single protein, but rather to the cytA27 molecule acting synergistically with one or more of the other proteins from the Bt subsp. *israelensis* inclusion body.

Although some disagreement remains about the mosquitocidal toxicity of the cytA27 protein, there is a broad agreement that the cytA27 protein from Bt subsp. *israelensis* accounts for most if not all of the cytolytic and haemolytic activities against a wide range of cell types *in vitro*. This property distinguishes the cytA27 protein from the majority of *cry* proteins whose toxicity *in vitro* and *in vivo* is limited to insect cells. Cyt proteins are classified into one of the two main categories of Bt proteins to distinguish them from the majority *cry* proteins. Since they are haemolytic, it is likely that cytA proteins are less specific in their action on insect cells compared to the other Bt proteins and their mechanism of action may be different from that of other proteins in the Bt family.

Thomas and Ellar (1983b) investigated the interaction of cytA27 protein from Bt subsp. *israelensis* with the lipids from cultured mosquito cells. They demonstrated that preincubation of protoxin with mosquito plasma membrane phospholipid components, such as unsaturated phosphatidyl choline, caused the inactivation of toxicity both *in vitro* and *in vivo*. They suggested that the

cytolytic protoxin may bind preferentially to certain plasma membrane phospholipids. Subsequently, Drobniowski and Ellar (1988a) demonstrated that in the absence of other proteins, cytA27 can make phospholipid vesicles containing unsaturated fatty acids permeable to $^{86}\text{RbCl}$.

Knowles *et al.* (1989) studied the formation of single ion channels in a planar lipid bilayer by adding the cytA24 protein to a bilayer made up of 1-palmitoyl-2-oleoylphosphatidylethanolamine (PE). Measurement of current indicated that the cytA proteins form cation-selective channels in the bilayer; the channels are permeable to K^+ , Na^+ but not to N-methylglucamine or Cl^- . The frequency and number of channel openings were reduced greatly in the presence of divalent cations (Ca^{2+} and Mg^{2+}) leading the authors to speculate that channel formation is inhibited by divalent cations. The underlying mechanism of this phenomenon remains unknown.

Limited knowledge about the structure of the cytA proteins is available. Preliminary crystallographic structural analysis suggested that the cytA27 protein contains a parallel or anti-parallel helix bundle as a major structural unit. This structure may facilitate insertion into cell membranes (McPherson *et al.*, 1987). A secondary structural model of cytA27, generated using a computer algorithm method, has been presented by Ward *et al.* (1988). This model indicates the

existence of several hydrophobic regions. Extensive, site-directed mutagenesis studies indicated that residues located on helical and turn/coil regions of the predicted structural model are necessary for an interaction of the protein with phosphatidyl choline liposomes. To investigate the functional role of the hydrophobic segments of the cytA proteins, Gazit and Shai (1993) characterized spectroscopically the two synthetic peptides corresponding to the putative amphiphilic α -helix regions of cytA27 protein (amino acids 50-71 and 110-131). Their results suggested that the two hydrophobic segments participate in the specific interaction with the membrane and might facilitate pore formation by the protein.

The mechanism of cell-membrane interaction proposed for the cytA proteins is different from that of the *cry* proteins as mentioned above (Gill *et al.*, 1992). The binding of cytA proteins to the membranes begins with the interaction between the proteins and membrane phospholipids, followed by aggregation of the protein molecules on the cell membrane, osmotic imbalance and cell lysis. Due to the specific features in their structure and biological activity, cytA proteins were chosen in our study of Bt mode of action to investigate the interaction of these proteins with phospholipid membranes.

Most previous studies (Thomas and Ellar, 1983a; Hurley *et al.*, 1985;

Delécluse *et al.*, 1991; Knowles *et al.*, 1989; Drobniowski and Ellar, 1988) have not compared the effects of cytA27 and cytA24 proteins from Bt subsp. *israelensis*. They used either cytA27 (Thomas and Ellar, 1983a; Hurley *et al.*, 1985; Delécluse *et al.*, 1991) or cytA24 derived from trypsin digested cytA27 protein (Knowles *et al.*, 1989). Only one study compared the mosquitocidal toxicity of purified cytA24 and cytA27 proteins (Chilcott and Ellar., 1988) and no work has been undertaken comparing the modes of actions of cytA24 and cytA27 proteins. We present a detailed comparison for the first time.

7. Objectives of the present study

The increasing importance of *Bacillus thuringiensis* proteins in pest control emphasises the need to understand the fundamental mechanism of Bt proteins as bioinsecticides. There is an urgent requirement to study Bt proteins mode of action, especially to provide precise information at the cellular and molecular levels. The present study focuses on one of the steps involved in insecticidal activity *viz.* protein-membrane interaction. We have chosen the cytA proteins from Bt subsp. *israelensis* which have broad cytolytic activity, to undertake a study of the interaction of a Bt protein with an artificial phospholipid membrane.

The goals of the present study are to gain information about Bt's mode of action, to investigate the effect of the cytA proteins on phospholipid bilayer integrity, to investigate the binding kinetics and factors affecting cytA binding activity, and to compare the interactions of the cytA27 and cytA24 proteins from Bt subsp. *israelensis* with phospholipid membranes.

Materials and Methods

1. Materials:

Bacillus thuringiensis subsp. *israelensis* strain was obtained from the Institut Pasteur (Paris, France). The fermentation of Bt was undertaken by the Cell Physiology Group at the Institute for Biological Sciences (NRC, Canada). Egg-PC (phosphatidylcholine) in chloroform (estimated molecular weight 750), calcein and CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) were purchased from Sigma (St. Louis, MO, USA). FITC-dextran (Fluorescein Isothiocyanate - dextran, M.W. 3,000 and 10,000) was obtained from Molecular Probes Inc. (Eugene, OR, USA) Sephadex G-50 and 75 were from Pharmacia LKB (Uppsala, Sweden). Acrylamide (purity >99.9%) was from Bio-Rad (Richmond, CA, USA).

2. *Bacillus thuringiensis* subsp. *israelensis* crystal isolation:

The first step in Bt protein studies is usually the isolation of the crystalline inclusion bodies. The crystals, which consist of Bt proteins, represent 20-30% of the cell dry weight.

B. thuringiensis subsp. *israelensis* cells were grown in 1 litre of tryptone soya broth medium at 28°C for 24 hours and were used to inoculate a 50-litre fermentation batch. Fermentation was continued until sporulation was complete,

requiring about two days. The fermentation suspension was collected, pelleted from the growth medium (10,000 rpm for 20 minutes), washed once with distilled water, then with 0.5 M sodium chloride and 0.1% Triton X-100, resuspended in water and stirred overnight at 4 °C. Under these conditions, the cells lysed efficiently, releasing parasporal crystals and spores. The crystals and spores were removed from the suspension by centrifugation (10,000 rpm for 20 minutes) and washed at least five times in water. The crystals were subsequently separated from spores and debris by buoyant density ultracentrifugation in 65% Renografin (a X-ray contrasting agent, Squibb Canada Inc., Montreal) at 15,000 rpm for 30 minutes. After centrifugation, the spores pelleted at the bottom of the tube. A band consisting of highly enriched parasporal crystal inclusions formed above the spores and under the surface layer which contains lipids and debris. The purity of crystals was examined using a phase contrast microscope; crystals appeared as diamond-shapes and bright, and spores appeared as bar-shaped objects. Pure crystals were washed with water 2-3 times and stored at 4°C. 10 µl of crystal suspension was solubilized in 1ml 0.1N KOH solution (pH 13.0) and the absorbance was monitored at 280 nm to estimate the approximate protein concentrations. The absorbances of 1 cm path length in 1 mg/ml of the cytA27 and cytA24 protein solution are approximately 0.96 and 0.86, respectively. These are calculated according to the

number of tyrosine (n_{tyr}) and tryptophan (n_{trp}) residues and by $(5,700 n_{\text{trp}} + 1,300 n_{\text{tyr}})/M$, where M is the molecular weight of the proteins (Cantor and Schimmel, 1980).

Purified crystals were solubilized in sample buffer for SDS-PAGE (3.4 g/l NaH_2PO_4 , 1.02 g/l Na_2HPO_4 , 10 g/l SDS, 1% β -mercaptoethanol, 0.15 g/l β -bromophenol blue and 0.36 g/l urea), and heated at 100°C for 3 minutes. The solubilized proteins were characterized by SDS-PAGE analysis. Polyacryamide SDS-gels with 10-15% gradient were run on a Pharmacia Phast electrophoresis system (Pharmacia LKB, Uppsala, Sweden). The preformed gels and other materials were supplied by Pharmacia. Gels were developed with Coomassie blue.

3. Isolation of the cytA27 and cytA24 cytolytic proteins from *Bacillus thuringiensis* subsp. *israelensis* crystals

Isolation of the cytA27 cytolytic protein from Bt subsp. *israelensis* was achieved by selective solubilization and ion exchange HPLC. The cytA27 protein isolation was carried out using a modification of the conditions described previously by Chilcott and Ellar (Chilcott and Ellar, 1988). About 8 mg of purified crystals were suspended in 5 ml 0.05 M $\text{Na}_2\text{CO}_3/\text{HCl}$, pH 9.5. 0.1M freshly made dithiothreitol (DTT) solution (in 0.05M $\text{Na}_2\text{CO}_3/\text{HCl}$ buffer, pH 9.5) was added to the

suspension to form a final concentration of 10 mM. The samples were incubated at 37°C for 60 minutes and then centrifuged at 12,000 rpm for 10 minutes in Eppendorf minifuge tubes. The supernatant was removed and filtered through 0.22 μm membrane filters (Millipore, Bedford, MA, USA). Further purification of cytA27 was achieved using a Mono Q anion-exchange column (Pharmacia, Uppsala, Sweden) connected to a Waters 600E HPLC system (Millipore, Milford, MA). The column was equilibrated with 0.01N NH_4HCO_3 , pH 8.5 (pH adjusted with ammonium hydroxide). After loading the sample, a gradient of 0.01 M to 0.8 M NH_4HCO_3 buffer (pH 8.5) was used to release the proteins bound to the column, at a flow rate of 1 ml/min. The collected fractions were subjected to a 10-15% SDS-PAGE analysis to identify the cytA27 protein and to check the purity of separation. The purified cytA27 protein fractions were frozen, lyophilized and stored at 4°C or -20°C for short or long term storage, respectively.

The cytolytic 24 kDa protein of Bt subsp. *israelensis* was obtained by tryptic digestion of the cytA27 protein and further purified on ion exchange HPLC. About 1 mg of freeze-dried cytA27 protein was dissolved in 2 ml 0.01 N NH_4HCO_3 (pH 8.5) and then incubated with trypsin (final concentration 200 $\mu\text{g}/\text{ml}$) at 37°C for 30 minutes. The digested sample was applied to a Protein Pak SP 5PW anion

exchange column (Waters, Millipore, Milford, MA, USA) which was equilibrated with 0.01 M NH_4HCO_3 , pH 8.5. The proteins were eluted by using a step-wise gradient of 0.01 N-0.8 N NH_4HCO_3 . The flow rate was 1 ml/min. The collected fractions were examined by SDS-PAGE analysis. The purified cytA24 protein fractions were lyophilized and stored at 4°C or -20°C for short or long term storage, respectively.

4. N-terminal amino acid sequencing:

The N-terminal sequencing was performed by Mr. David Watson (Institute for Biological Sciences, NRC, Canada) on a Model 475A protein sequencer (Applied Biosystem Inc., Foster City, CA, USA) by Automated Edman Degradation. The sequencer incorporated a model 470A gas phase sequencer equipped with an on-line model 120A PTH analyzer under the control of a model 900A control/data analysis module. About 0.1-0.5 nmol of proteins were required to carry out the analyses.

5. Mass spectral analysis:

The mass spectra of cytA proteins were determined by Dr. Makoto Yaguchi and Mr. David Watson at the Biotechnology Research Institute (National Research Council of Canada, Montreal, Canada). The protein samples were dialysed against

5% acetic acid and 50% acetonitrile overnight. A minimum 25 μ l of 0.1 mg/ml sample solution is needed to acquire the spectrum. A triple quadrupole mass spectrometer (the API III LC/MS/MS system, Sciex, Thornhill, Ontario, Canada) was used. The instrument has a mass to charge ratio (m/z) range of 0-2,400. The average molecular mass values of the proteins were calculated from the m/z peaks in the charge distribution profiles of the multiply charged ions.

The molecular masses of cytA27 and cytA24 proteins were also calculated using a programme named MacBioSepc/MacProMass (PE Sciex, Thornhill, Ontario, Canada) based on the nucleotide sequence of the cloned cytA27 gene.

6. Haemolysis experiment:

Rabbit blood was kindly donated by Dr. J. Phipps (Institute for Biological Sciences, NRC, Canada). Red blood cells (RBC) were washed twice in 50% (v/v) phosphate buffered saline (PBS) containing 2.5% glucose (w/v), using centrifugation at 4,500 rpm for 5 minutes to compact the cells. Finally, the cells were packed more tightly by a centrifugation at 10,000 rpm for 5 mins. A 0.1% (v/v) RBC suspension was made in 50% PBS (v/v) just before each experiment. 1,2,4,8 μ g/ml cytA proteins (final concentration) were used to haemolyse the RBC. All test samples were made up to a final volume of 1 ml. 0.1% RBC suspension

without addition of the proteins was used as the control for 0% release. 25 μ l 10% Triton X-100 was mixed with RBC suspension as a standard for 100% lysis. The samples were incubated at 4°C for 30 minutes and then were centrifuged at 2,000 rpm for 2 minutes. The amount of haemoglobin released was estimated by measuring the absorbance of the supernatant at 540 nm.

7. Fluorescence assay of the cytA protein-induced permeabilization of phospholipid vesicles

Large unilamellar vesicles (LUV) were prepared by extrusion. A solution of 5 mg of egg-phosphatidylcholine (egg-PC) in 50 μ l chloroform was dried with a stream of nitrogen gas to form a thin, transparent film in a 3 ml glass vial by rotary evaporation. Calcein powder was dissolved in H₂O to make a 80 mM solution and adjusted to pH 7.4 with 1 N NaOH. The dried lipid was suspended in 500 μ l of 80 mM calcein (pH 7.4) solution. The mixture was vortexed, frozen and thawed twice, and extruded through a stack of two 0.1 μ m pore size polycarbonate membrane filters (Nuclepore, Toronto, Canada) in Liposofast (Avestin, Ottawa, Canada). To remove untrapped calcein, 200 μ l of extruded vesicle mixture was then passed through a Sephadex G-50 column (1 \times 30 cm), eluted with a buffer containing 50 mM HEPES, 100 mM NaCl, 0.3 mM EDTA, adjusted to pH 7.4 by

1 N NaOH (Buffer A). Phospholipid concentrations were determined by inorganic phosphate assay (Ames, 1968).

Small unilamellar vesicles (SUV) were prepared by sonication. The initial lipid film was prepared as mentioned above for LUV. The dried lipid was suspended in calcein solution by vortex mixing. SUVs were obtained by sonication in an ice bath for 5 minutes on a Sonifier cell disruptor 185 (Branson Cleaning Equipment Company, Shelton, CT, USA). When required, extra sonication was performed until the solution became clear. Unentrapped calcein was removed by column chromatography on Sephadex G-50 (1 × 30 cm) using buffer A as described before.

In dextran release experiments, 5 mM FITC-dextran (M.W. 10,000) and 10 mM FITC-dextran (M.W. 3,000) solutions at pH 7.4 were used to prepare LUV replacing calcein in the procedure presented above.

Fluorophore Marker Release Experiments:

In a typical experiment, purified cytA27 and cytA24 proteins were dissolved in 0.01 N NH_4HCO_3 (pH 8.5). The exact concentrations of protein stock solutions were determined by measuring O.D. at 280 nm. The protein stock solutions were kept on ice during experiments. Fluorescence measurements were performed on an

SLM8000C fluorometer equipped with double monochromator (SLM Instruments Inc., Urbana, IL, USA). The cuvette was thermostated at 20°C with continuous stirring. At concentrations above 1 μ M, calcein fluorescence is self-quenched (Allen, 1984). This self-quenching property of calcein was used in the present experiments. CytA induced membrane permeability (release of calcein from the interior of the vesicles) was monitored by detecting the decrease in self-quenching (an increase in fluorescence) when calcein was released into the external solution and thus diluted. The rate of spontaneous calcein release was negligible. The initial concentration of calcein in vesicles was 80 mM. For the fluorescence measurements, the excitation wavelength was set at 494 nm and the emission was recorded at 520 nm. The extent of release of fluorophore marker from vesicles and the release kinetics were determined at various combinations of protein and lipid concentrations. 1 μ l to 20 μ l of vesicle suspension made from 0.88 mM lipid was added to 1.5 ml of buffer A in a 1 \times 1 cm cuvette. Subsequently, the reaction was initiated by addition of 5-370 nM protein into the stirred lipid vesicle suspension. The signal was monitored until a steady value was reached. The fluorescence signal from intact vesicles in buffer prior to the addition of protein was taken as the standard for 0% release. 100% of release was achieved by detergent lysis, 10-20 μ l of 10% Triton X-100 was added to the cuvette. This value was used for

normalization of the data. All experiments were carried out in the presence of EDTA to eliminate the effect of divalent ions on calcein fluorescence.

The pH dependence of calcein release was measured. The experiments were conducted in 100 mM NaCl buffered with 10 mM citrate (pH 4), 10 mM CAPS (pH 10), and 10 mM HEPES (pH 7.4).

CytA-induced FITC-dextran release was tested by using 32-130 nM cytA24 for releasing FITC-dextran of molecular weight 10,000 and 260 nM cytA24 for FITC-dextran of molecular weight 3,000.

8. Release mechanism:

To determine the mechanism of release, the fluorescence ratios (before and after addition of Triton) of the vesicles remaining after incubation with proteins were measured. Constant concentration of lipid vesicles containing calcein were incubated with proteins at different concentration. 0.69 - 3.1 μ M of cytA24 or cytA27 proteins were mixed with 0.16 mM LUV suspension in buffer A (see pp. 31-32) at a total volume of 0.5 ml. The mixture was incubated for 15 minutes with frequent vortexing. A sample without addition of protein was used as a control.

Intact vesicles were then separated from free calcein, which was released during incubation, by column chromatography. The samples were applied to disposable

Sephadex G-25M columns (1.5×5 cm, Pharmacia, Uppsala, Sweden), then eluted with buffer A (50 mM HEPES, 100 mM NaCl, 0.3 mM EDTA, pH7.4). The first 0.5 ml eluted of the intact vesicle fraction was collected and subjected to fluorescence measurements. From this fraction the fluorescence of 200 μ l was measured in the absence (F_0) and presence (F_{100}) of 3 μ l Triton X-100. Additionally, the fluorescence of a separate 200 μ l fraction was tested in the presence of 10 μ l 10 mM CoCl_2 solution (F_c). Co^{2+} was chosen as a calcein fluorescence quencher to eliminate the fluorescence signal from free calcein present as a contaminant. The fluorescence ratios of the remaining intact vesicles at each protein to lipid ratio for both cytA27 and cytA24 proteins were calculated from the formula $F_c/(F_{100}-F_0+F_c)$. The fluorescence ratios obtained from these data were plotted versus corresponding protein/lipid ratios.

The value of the fluorescence ratio obtained from a control experiment (without addition of protein) will be the predicted fluorescence ratio at different protein to lipid ratios for the all-or-none release model.

Additional experiments were carried out which enable us to predict the dependence of the fluorescence ratios on the protein to lipid ratios in the event that a graded release occurs. A standard curve was constructed to develop a relationship between interior calcein concentrations and fluorescence ratios in the absence of

proteins. The fluorescence of LUVs, prepared as described above and containing 5 to 80 mM calcein were measured before and after detergent lysis. Fluorescence ratios which are simply pre and post Triton in this case were plotted against intra-LUV calcein concentration as the standard curve. Separate fluorescence ratios were then measured for vesicles in the presence of different amount of proteins. At each protein lipid ratio, the extent of cytA24 or cytA27 induced calcein release was determined by measuring the fluorescence signals before (F_p) and after (F_{100}) addition of 3 μ l 10% Triton X-100 into 200 μ l protein and lipid mixture after incubation without separation of intact vesicles. From these data, the percentage of release of calcein from vesicles Q ($Q = [F_p - F_0]/[F_{100} - F_0]$, F_0 is the fluorescence from LUVs before adding proteins) at each protein lipid ratio and the percentage of remaining calcein in the vesicles ($1 - Q$) were known. The remaining calcein concentrations at each protein lipid ratio were predicted from the equation: $C = (1 - Q) \times C_0$ (C_0 , calcein concentration before addition of protein). The fluorescence ratios under these calcein concentrations were obtained from the standard curve and used as predicted values for graded model.

The predicted values for both models of release were then plotted and compared with experimental results.

9. Tryptophan fluorescence quenching by acrylamide or iodide.

Two kinds of tryptophan fluorescence quenchers acrylamide and potassium iodide were employed in the study of cytA binding to the lipid vesicles.

SUVs were prepared as described above. 7.4 M acrylamide, 4 M KI and KCl were prepared in water adjusted to pH 7.4 with buffer A. A small amount of $\text{Na}_2\text{S}_2\text{O}_3$ was used to keep iodide in its reduced form. 210 μl of 3.53 μM cytA27 or 3.97 μM cytA24 protein solution in a 0.3×0.3 cm cuvette were titrated with 7.4 M acrylamide until the fluorescence spectrum became invariant. This experiment was performed in the absence and in the presence of lipid (1.65 mM SUV). Similar experiments were performed with KI using 4 M KCl to maintain constant ionic strength.

Results

1. *Bacillus thuringiensis* protein crystal preparation:

About 1.2 g crystals of Bt subsp. *israelensis* were obtained as described in Materials and Methods. Purified crystals were used for the isolation of the cytA27 cytolytic protein from Bt subsp. *israelensis*.

2. CytA27 and cytA24 protein purification and characterization:

a. Selective solubilization of Bt subsp. *israelensis* crystals

It is known from a previous study (Federici et al., 1990) that Bt subsp. *israelensis* crystals contain a mixture of 135, 128, 65 and 27 kDa proteins. The solubility properties of crystals were examined by dissolving crystal suspensions in different buffers and by subsequent SDS-PAGE analysis. The crystal suspensions were solubilized in buffer A: 0.05 N CAPS (pH 11.5); or B: 0.05 N NaHCO₃ (pH 9.5); or C: 0.05 N NaHCO₃, 0.01 M DTT (pH 9.5). The SDS-PAGE gel in Figure 1 shows the protein composition of solubilized Bt subsp. *israelensis* crystals in different buffers. The crystals dissolved in high pH 11.5 buffer (Lane 2) or lower pH 9.5 containing reducing agent DTT (Lane 5) but not in pH 9.5 buffer in the absence of reducing agent (Lane 3). The 130, 65 and 27 kDa proteins are seen when the crystals dissolved in pH 11.5 buffer as

well as a 35 kDa and a 30-35 kDa protein (lane 2) which are the proteolytic products of the 130 kDa and 65 kDa proteins (Federici et al., 1990). In pH 9.5 buffer with DTT, only the 27 kDa and 130 kDa protein dissolved (Lane 5), with the 65, 35 kDa and some 27 kDa proteins remaining in the pellet (Lane 6). Thus, the dissolution of the protein components of Bt subsp. *israelensis* crystals is highly dependent on buffer and pH.

b. Isolation of the cytA27 protein

Ion-exchange chromatography was employed to separate the 27 kDa protein from other solubilized Bt subsp. *israelensis* proteins (see Methods and Materials). Figure 2 shows the elution profile of cytA27 separation by ion-exchange HPLC. Four major peaks were observed when absorbance was measured at 280 nm. The protein components of each peak were determined by SDS-PAGE analysis (Figure 3, gel 1). Peak c of the chromatogram (Figure 2) corresponds to lane 4 on the gel and contains the 27 kDa protein with no evidence of other bands. Peak d consists of the 130 kDa protein (lane 5) and peak b is a 35 kDa protein (Lane 3). The first huge peak (peak a) is the result of DTT absorbance (lane 2). N-terminal sequencing was used to confirm the SDS-PAGE results. The N-terminal sequence of the 27 kDa protein from the peak c fraction in Figure 2 and amino acid sequence predicted from the

nucleotide sequence of the cloned Bt subsp. *israelensis* cytA27 gene are presented below.

1. N-terminal amino acid sequence of the protein from peak c (Figure 2):

MENLN H?PLE DIKVN PWKTP

2. CytA27 protein N-terminal sequence predicted from DNA sequence:

(Waalwijk *et al.*, 1985)

MENLN HCPLE DIKVN PWKTP

The N-terminal sequence of the isolated 27 kDa protein agrees well with the sequence predicted from the nucleotide sequence of the cloned cytA27 gene.

c. Production of cytA24 by limited proteolysis

The cytA24 protein was derived by the tryptic digestion of isolated cytA27 of Bt subsp. *israelensis* and purified by ion-exchange HPLC. Figure 4 is the chromatogram obtained during cytA24 protein purification. Only two significant protein peaks were detected with retention times (R_t) of 5 and 50 minutes. SDS-PAGE analysis results indicated that peak b contained a protein with expected molecular weight of 24 kDa (Figure 3, gel 2). The peak eluted at the beginning of the run was found to contain trypsin by comparing the elution time with that from a run of trypsin alone (Figure 5).

The N-terminal sequence of the fraction containing the 24 kDa protein was

determined and compared with the N-terminal of the intact cytA27 protein amino acid sequence, predicted from the cloned cytA27 gene nucleotide sequence, by referring to Figure 6.

The N-terminal amino acid sequence of the 24 kDa protein is

VEDPN EINNLSINE IDNPN

(N)

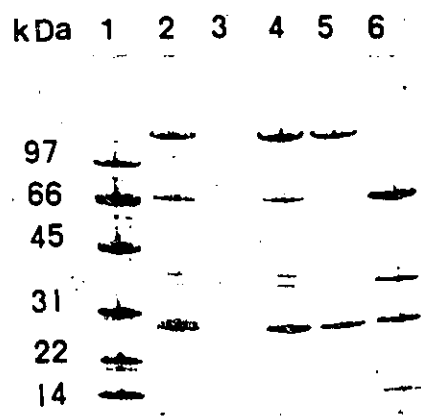
?

The comparison indicates that the 24 kDa protein is produced by the removal of 30 amino acids from the N-terminal of the cytA27 protein. The cleavage occurs between an arginine (R) and a valine (V) residue. The amino acid sequence of the cytA24 protein is shown in Figure 7.

Shown in Figure 8 and 9 are the electrospray mass spectra of cytA27 and cytA24, respectively. Both proteins demonstrate very similar spectral behaviour, producing a charge distribution profile in the high m/z range (2000-2400) of the instrument. The average molecular weights of the cytA24 and cytA27 proteins are determined as $23,856.87 \pm 1.34$ and $27,348.76 \pm 1.85$, respectively. These values are in accord with the SDS-PAGE determined molecular weights and are also very close to the calculated molecular weights 27,342.2261 amu for cytA27 (Figure 6) and 23,855.1519 amu for cytA24 (Figure 7). The C-terminal amino acid of cytA27 is predicted from the above results and calculations to be leucine(L) (see Figure 6 and 7).

Figure 1. SDS - PAGE gel of solubilization of *Bacillus thuringiensis* subsp. *israelensis* crystals in different buffers.

- Lane 1, Molecular weight standard (Phosphorylase b, 97,400; Bovine serum albumin, 66,200; Ovalbumin, 45,000; Carbonic anhydrase, 31,000; Soybean trypsin inhibitor, 21,500; Lysozyme, 14,400).
- Lane 2, B.t. subsp. *israelensis* crystal proteins dissolved in buffer A: 0.05 N CAPS (pH 11.5).
- Lane 3, B.t. subsp. *israelensis* crystal proteins dissolved in buffer B: 0.05 N Na₂CO₃/HCl buffer (pH 9.5)
- Lane 4, B.t. subsp. *israelensis* crystals dissolved in buffer C: 0.05 N Na₂CO₃/HCl buffer (pH 9.5) with 0.01M DTT.
- Lane 5, The supernatant of B.t. subsp. *israelensis* crystal suspension in 0.05 N Na₂CO₃/HCl buffer (pH 9.5) with 0.01M DTT.
- Lane 6, The pellet of B.t. subsp. *israelensis* crystal suspension in 0.05 N Na₂CO₃/HCl buffer (pH 9.5) with 0.10M DTT.



0
:

Figure 2. Purification of the cytA27 protein from solubilized *B. thuringiensis* subsp. *israelensis* crystal proteins using ion-exchange HPLC on a Mono-Q column.

Crystals dissolved in 0.05M Na₂CO₃/HCl, 0.01M DTT (pH 9.5). The column was equilibrated with 0.01M NH₄HCO₃ (pH 8.5), 0.8M NH₄HCO₃ (pH8.5) as eluting buffer.

Filename: C0279
Operator: F.H

Sample: Cyt AS Isolatlin
Channel: WATERS 441 U.V.
Acquired: 05-MAR-93 15:05
Inj Vol: 7.00
Method: C:\MSK\DATA\VELLAR

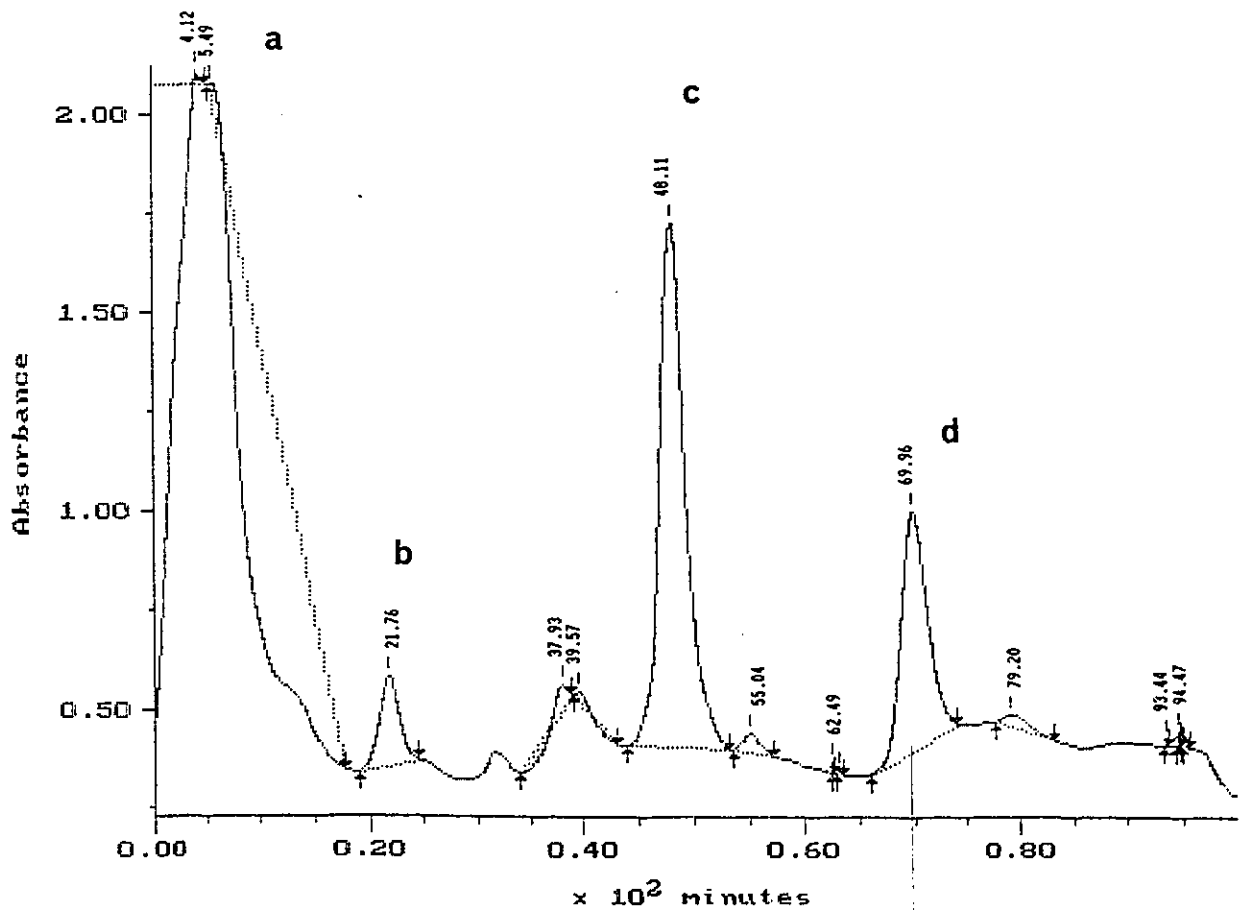


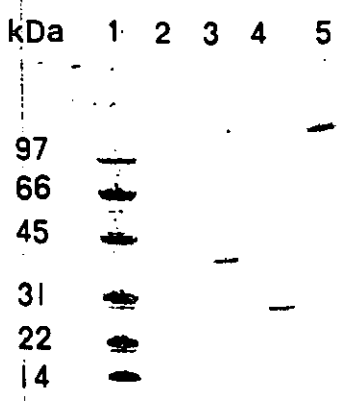
Figure 3. SDS-PAGE of the cytA27 protein and cytA24 protein separation.

Gel 1: The cytA27 protein identification.

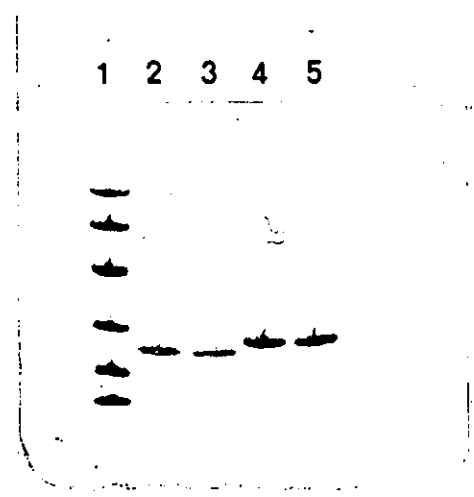
Lane 1, molecular weight standard (same as used in Figure 1). Lane 2, peak a in Figure 2, DTT peak. Lane 3, peak b in Figure 2, 35 kDa protein. Lane 4, 27 kDa protein, peak c in Figure 2. Lane 5, 130 kDa protein, peak d in Figure 2.

Gel 2: The purified cytA27 and cytA24 proteins.

Lane 1, molecular weight standard (same as used in Figure 1). Lane 2,3, purified 24 kDa protein from trypsin digestion of the cytA27 protein, peak b in Figure 3. Lane 4,5, The purified 27 kDa protein from *B.t. subsp. israelensis*, peak c in Figure 2.



1



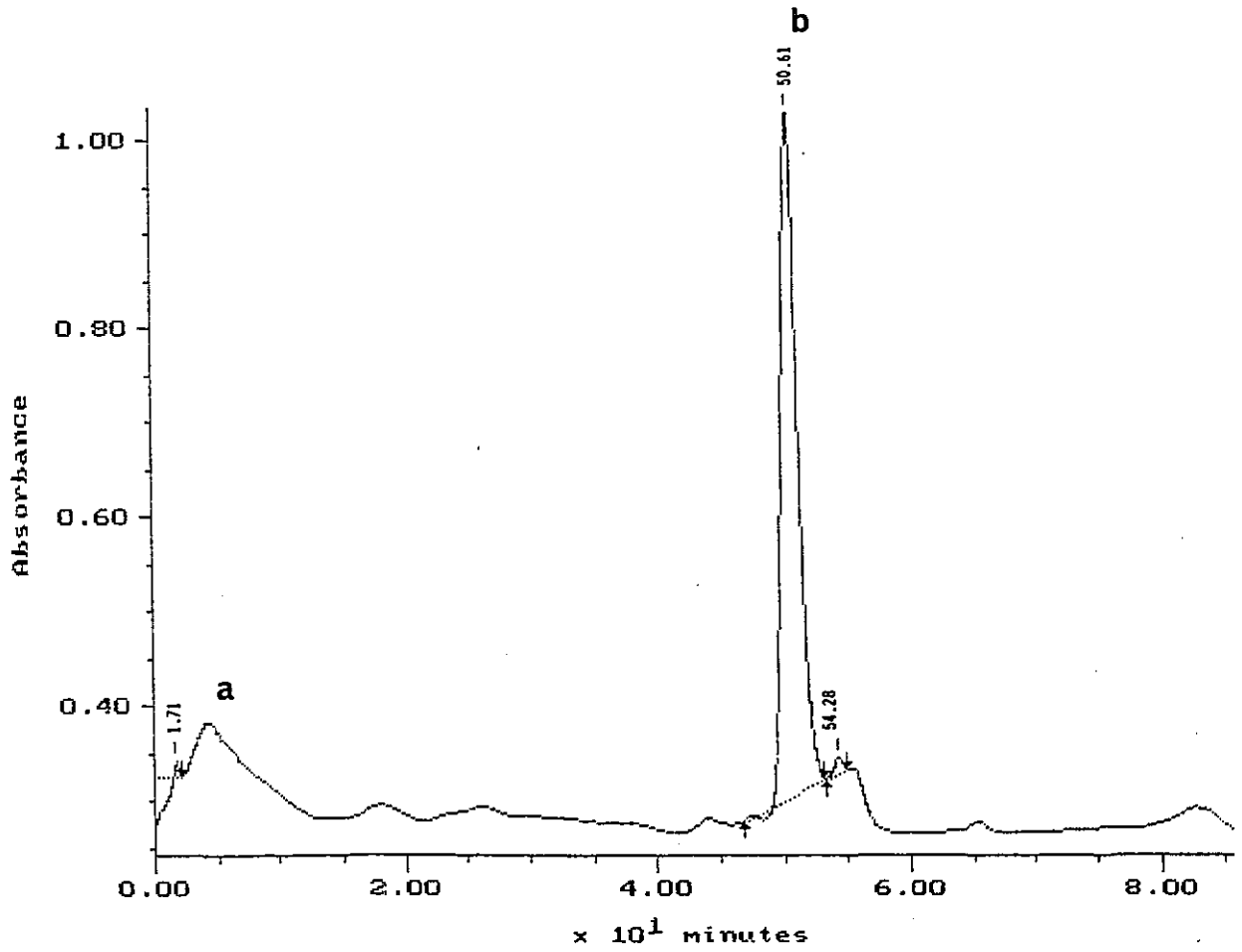
2

Figure 4. Chromatogram of the cytA24 protein isolation by ion-exchange HPLC.

Using a Protein Pak SP 5PW column equilibrated with 0.01M NH_4HCO_3 (pH 8.5), 0.8 M NH_4HCO_3 (pH8.5) as eluting buffer.

File name: C0342
Operator: F.H

Sample: C0337 trypsin
Channel: WATERS 441 U.V.
Acquired: 16-APR-93 13:41
Inj Vol: 3.00
Method: C:\MAXDATA\VE1



4
:

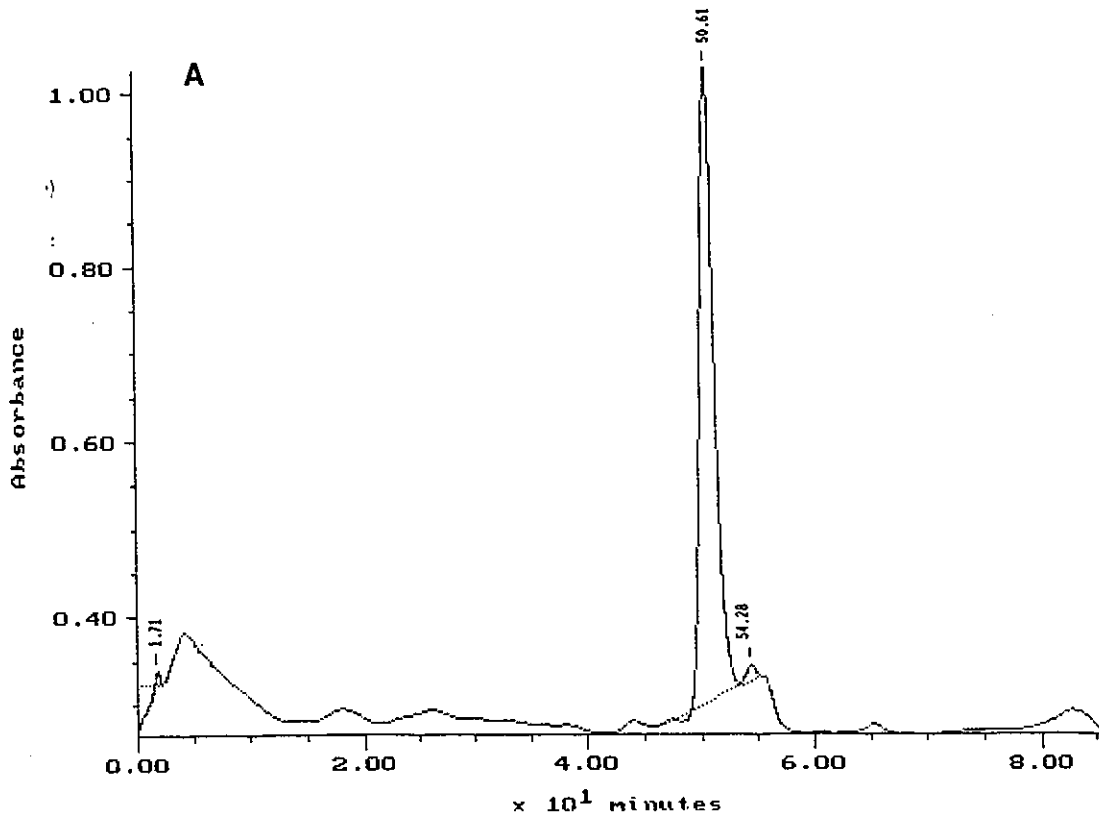
Figure 5A. Chromatogram of the cytA24 protein isolation by ion-exchange HPLC

Figure 5B. Elution profile of trypsin on a Protein Pak SP 5PW column

Elution conditions are the same as described for cytA24 isolation.

Filename: C0242
Operator: F.H

Sample: C0337 Trypsin
Acquired: 16-APR-93 13:41
Channel: WATERS 441 U.V.
Method: C:\MAXDATA\T1\1



Filename: C0289
Operator: F.H

Sample: Trypsin alone
Acquired: 15-APR-93 15:45
Channel: WATERS 441 U.V.
Method: C:\MAXDATA\T1\1

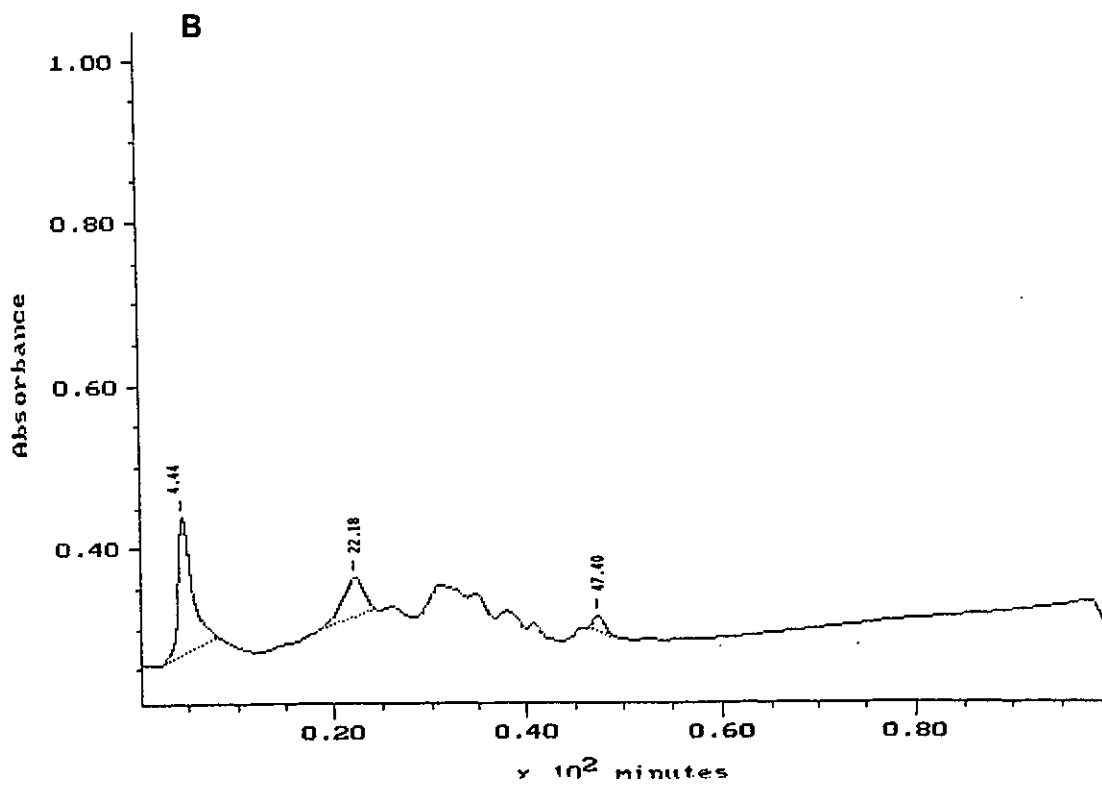


Figure 6. The Predicted amino acid sequence and calculated molecular weight of cytA27

Amino acid sequence is deduced from the nucleotide sequence of Bti cytA gene.

Cyt A27

↓

MENLNHCPLEDIKVNPKTPQSTARVITL[↓]RVEDPNEINNLLSINEIDNPNYILQAIMLANAFQNALVPTSTDFGDALRFS
 -----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
 10 20 30 40 50 60 70 80

MPKGLEIANTITPMGAVVSYVDQNV[↓]TQTNNQVSMINKVLEVLKTVLGV[↓]LSGSVIDQLTAAVTNTFTNLNTQKNEAWIF
 -----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
 90 100 110 120 130 140 150 160

WGKETANQNTYNTYNVLF[↓]AIQNAQTGGVMYCVPVGF[↓]EIKVSAVKEQVLFF[↓]TIQDSASYNVNIQSLKFAQPLVSSSQYPIAD
 -----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
 170 180 190 200 210 220 230 240

L[↓]TSAINGTL
 -----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
 250 260 270 280 290 300 310 320

N-Terminal Group : Hydrogen

C-Terminal Group : Free Acid

MH+ Monoisotopic Mass = 27324.9365 amu

HPLC index = 779.60

MH+ Average Mass = 27342.2261 amu

Bull & Breese value = -23660

Isoelectric Point (pI) = 5.8

Elemental composition: **C 1221 H 1931 N 316 O 378 S 8**

User-Defined Amino Acid Residues:

None

Amino Acid Composition for --> Cyt A27

Abbr.	#	Name	Abbr.	#	Name
A	20	Alanine	N	27	Asparagine
B	0		O	0	
C	2	Cysteine	P	11	Proline
D	9	Aspartic Acid	Q	16	Glutamine
E	11	Glutamic Acid	R	3	Arginine
F	10	Phenylalanine	S	16	Serine
G	10	Glycine	T	22	Threonine
H	1	Histidine	U	0	
I	18	Isoleucine	V	25	Valine
J	0		W	3	Tryptophan
K	10	Lysine	X	0	
L	22	Leucine	Y	7	Tyrosine
M	6	Methionine	Z	0	

Figure 7. The predicted amino acid sequence and calculated molecular weight of cytA24

30 amino acids removed from N-terminal of the cytA27 protein amino acid sequence according to the N-terminal sequencing results.

CytA24

VEDPNEINNLLSINEIDNPNYILQAIMLANAFQNALVPTSTDFGDALRFSPKGLEIANTITPMGAVVSYVDQNVQTQNN
 -----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
 10 20 30 40 50 60 70 80

QVSVMINVKVLEVLKTVLGVLSGSGVIDQLTAAVTNTFTNLNTQKNEAWIFWGKETANQTNQTYNVLFQAIQNAQTGGVMYC
 -----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
 90 100 110 120 130 140 150 160

VPVGFEEKVSAVKEQVLFFTIQDSASYNVNIQSLKFAQPLVSSSQYPIADLTSAINGTL
 -----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
 170 180 190 200 210 220 230 240

N-Terminal Group : Hydrogen

C-Terminal Group : Free Acid

MH+ Monoisotopic Mass = 23840.1292 amu

HPLC index = 717.00

MH+ Average Mass = 23855.1519 amu

Bull & Breese value = -22460

Isoelectric Point (pI) = 5.6

Elemental composition: **C 1068 H 1682 N 271 O 334 S 6**

User-Defined Amino Acid Residues:

None

Amino Acid Composition for --> CytA24

Abbr.	#	Name	Abbr.	#	Name
A	19	Alanine	N	24	Asparagine
B	0		O	0	
C	1	Cysteine	P	8	Proline
D	8	Aspartic Acid	Q	15	Glutamine
E	9	Glutamic Acid	R	1	Arginine
F	10	Phenylalanine	S	15	Serine
G	10	Glycine	T	19	Threonine
H	0	Histidine	U	0	
I	16	Isoleucine	V	23	Valine
J	0		W	2	Tryptophan
K	8	Lysine	X	0	
L	19	Leucine	Y	7	Tyrosine
M	5	Methionine	Z	0	

Figure 8. Electrospray mass spectrum and report of the cytA27 protein

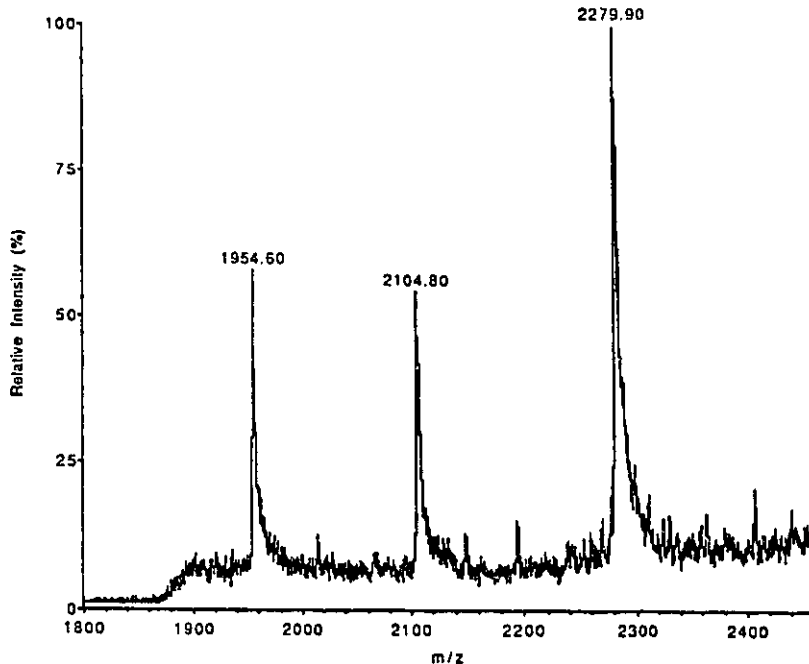
The instrument has a mass to charge (m/z) ratio range of 0 to 2,400. Samples were introduced by injection. Showing multiply charged ion series.

Profile Q1SCAN

DI 0
 ISV 5200
 IN 650
 CR 100
 R0 30
 M1 1000
 RE1 120.5
 DM1 0.050
 R1 28.5
 L7 -4.0
 R2 -4.0
 M3 1000
 RE3 122.5
 DM3 -0.080
 R3 -4.0
 L9 -2.50
 FP -2.50
 MU -3600
 CC 1
 CG Off
 DIV -0.645
 CGT 6.1
 ISC 6.6

FH BT C0399 28K - 5/18/93 - 2:43 PM
 FH BT C0339 28K
 3 peaks

299,990



Automated HyperMass Info for FH BT C0399 28K - PART 1

FH BT C0339 28K

Criteria used for HyperMass Method:

Primary Charge Agent: H, 1.0079 mass, 1.0000 charge, Agent Gained

Tolerance for peak estimates: 0.50

Peak threshold: 31,500 (10.5%)

Minimum peak width: 0.40

Scan step size: 0.10

Number of peaks: 3

HyperMass analysis completed Tuesday, May 18, 1993, 2:54:09 PM

```
=====
```

Actual peak Intensity	Pred. peak	Charge	Compound mass	
1954.60	174,220	1954.34	14	27,350.29
2104.80	162,158	2104.60	13	27,349.30
2279.90	299,990	2279.90	12	27,346.71

Avg. compound mass 27,348.76

Std. Deviation: 1.85

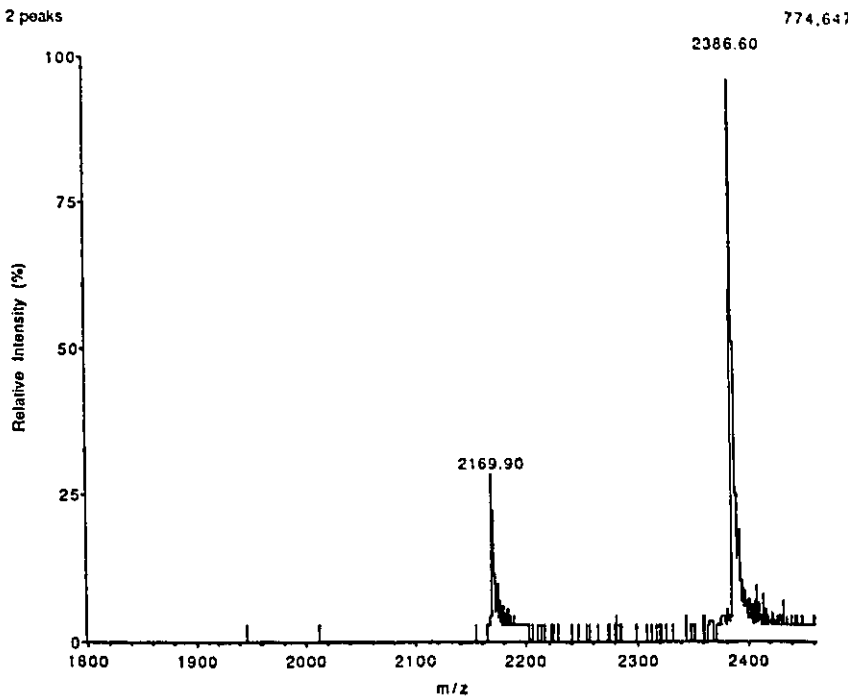
3 Estimates of compound mass

Figure 9. Electrospray mass spectrum and report of the cytA24 protein

The instrument has a mass to charge (m/z) ratio range of 0 to 2,400.

+Profile Q1SCAN

DI 0 FH BT C0344 24K - 5/18/93 - 2:34 PM
 ISV 5200 FH BT C0344 24K
 IN 650 2 peaks
 CR 100
 R0 30
 M1 1000
 RE1 120.5
 DM1 0.050
 R1 28.5
 L7 -40
 R2 -40
 M3 1000
 RE3 122.5
 DM3 -0.080
 R3 -40
 L9 -250
 FP -250
 MU -3600
 CC 10
 CG Off
 DIV -0.117
 CGT 6.1
 ISC 10.9



Automated HyperMass Info for FH BT C0344 24K - PART 1

FH BT C0344 24K

Criteria used for HyperMass Method:

Primary Charge Agent: H, 1.0079 mass, 1.0000 charge, Agent Gained

Tolerance for peak estimates: 0.50

Peak threshold: 80,000 (10.3%)

Minimum peak width: 0.40

Scan step size: 0.10

Number of peaks: 2

HyperMass analysis completed Tuesday, May 18, 1993, 2:39:39 PM

Actual peak	Intensity	Pred. peak	Charge	Compound mass
2169.90	218,717	2169.73	11	23,857.81
2386.60	774,647	2386.60	10	23,855.92

Avg. compound mass 23,856.87

Std. Deviation: 1.34

2 Estimates of compound mass

3. Haemolysis experiments:

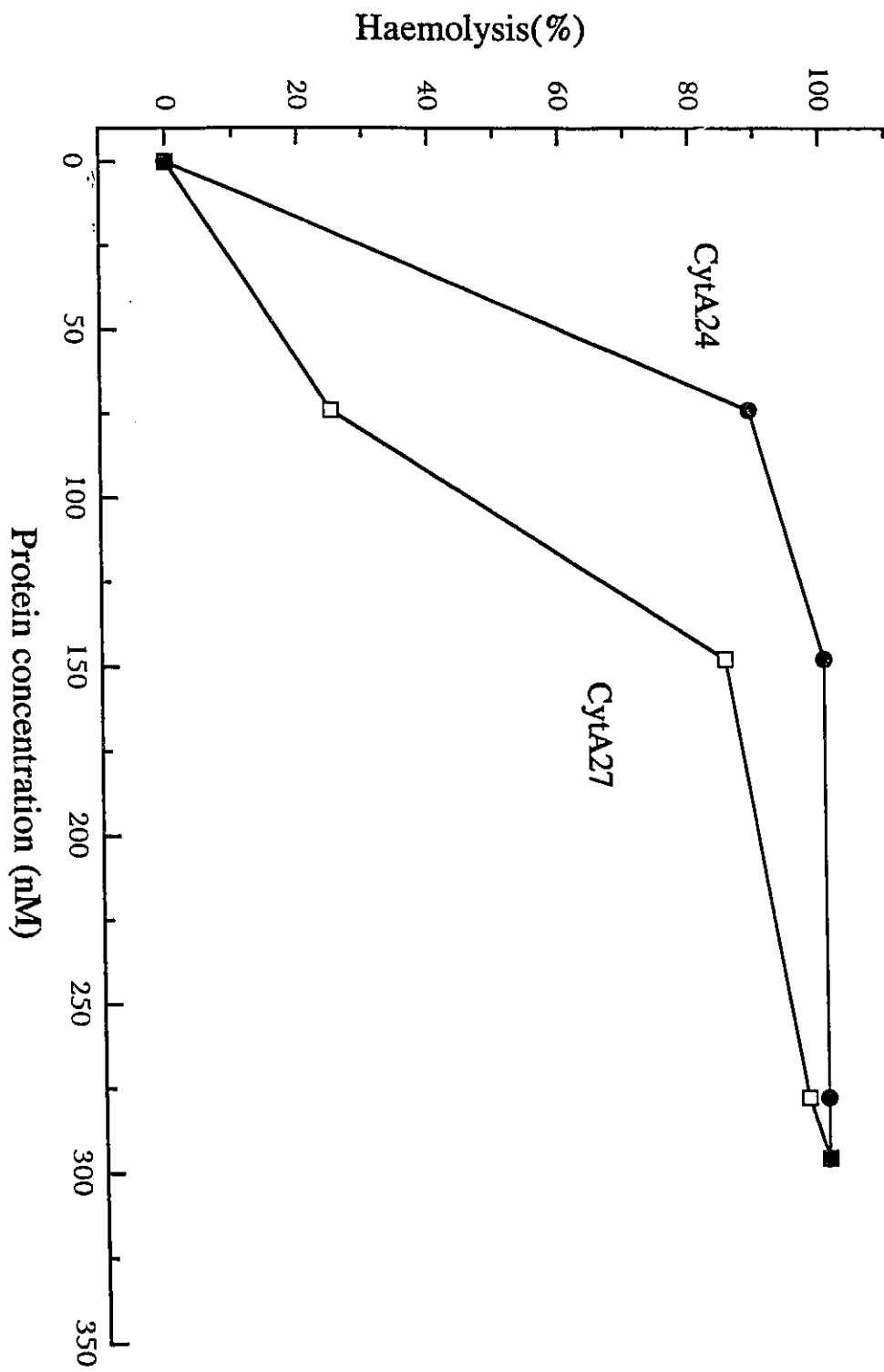
CytA27 and cytA24 proteins were assayed for haemolytic activity on red blood cells (see Materials and Methods). The haemolytic activities were measured by estimating the percentage of haemoglobin release, haemolysis % = $(O.D._{540P} / O.D._{540C}) \times 100$ ($O.D._{540P}$ is the absorbance at 540 nm for each protein concentration, $O.D._{540C}$ is the absorbance at 540 nm of 100% lysis in the presence of Triton X-100). Both cytA27 and cytA24 lysed erythrocyte cells and had enhanced haemolytic activities with increased protein concentrations (Figure 10). This is consistent with previous observations that the cytA proteins are haemolytic (Chilcott *et al.*, 1984, Delécluse *et al.*, 1991). Complete haemolysis occurred with more than 150 nM of cytA24 and more than 300 nM of cytA27 proteins; moreover at the same lower concentrations, cytA24 caused a higher degree of haemolysis than the cytA27 protein.

4. CytA27 and cytA24 induced release of fluorescence markers from lipid vesicles:

Figure 11 shows typical time course curves of the cytA27 protein induced release of calcein from egg PC vesicles. The release was measured as an increase in fluorescence and the release kinetics were determined from the data at a variety of protein and lipid ratios. The release curves could be fitted well

4)
:

Figure 10. Haemolytic activities of the cytA24 and cytA27 proteins

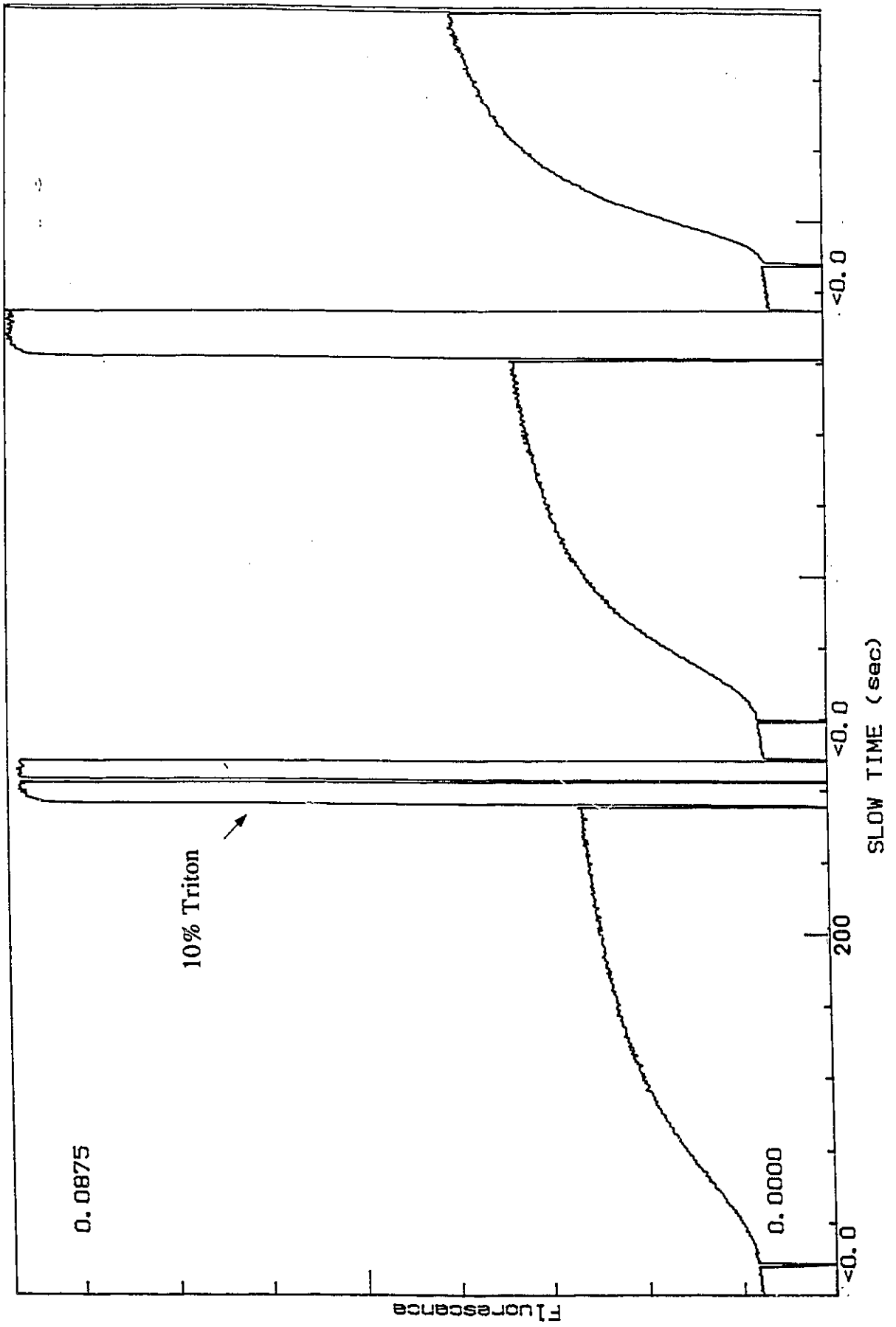


by a one-exponential equation $F(t) = (F_{\max} - F_0)(1 - \exp(-kt))$; where k is the release rate constant, and F_{\max} and F_0 are the maximum and initial fluorescence, respectively. For better fitting, an initial lag period of about 60s was excluded from the fittings. Figures 12, 13 and 14 show the leakage curves obtained with the cytA27 protein at three different pH values *viz.* 4, 7.4, and 10, respectively. Rate constants were obtained by fitting each curve and are listed in Table 3. In Figure 15, we have plotted these rates as a function of pH. There is an increase in release rate when the pH is decreased from 7.4 to 4, while a significant decrease is observed when the pH is increased to 10.0. At each pH, an increase in the protein (cytA27) concentration results in an enhanced release rate constant. CytA24-induced calcein leakage was studied at pH 7.4; leakage curves are shown in Figure 16 and rate constants in Table 3. At this pH no significant differences of release rates were observed between cytA27 and cytA24 proteins (Table 3).

The release of vesicle contents induced by cytA proteins was further characterized by the extent of release as a function of protein and lipid ratios in a series of experiments using two kinds of lipids vesicles, LUV and SUV. Figure 17 summarizes the results of cytA27 and cytA24 protein induced calcein release from LUV at 5 different protein concentrations and 3 lipid concentration levels. With increasing protein-lipid ratio, the extent of release increases until a

Figure 11. Typical time course curves of calcein leakage from egg PC vesicles induced by cytA proteins.

25, 50, 100 nM cytA27 were added to 6 μ M PC-LUV. The calcein leakage was monitored fluorometrically as a function of time until a saturated value was reached. 10 μ l - 20 μ l 10% Triton X-100 were added to reach 100% release.



)

:

Figure 12. Kinetics of the cytA27 protein induced calcein release from PC-LUV at pH 4.0

Protein concentrations ranging from 5 to 50 nM were used to combine with 1.2 μ M lipid.

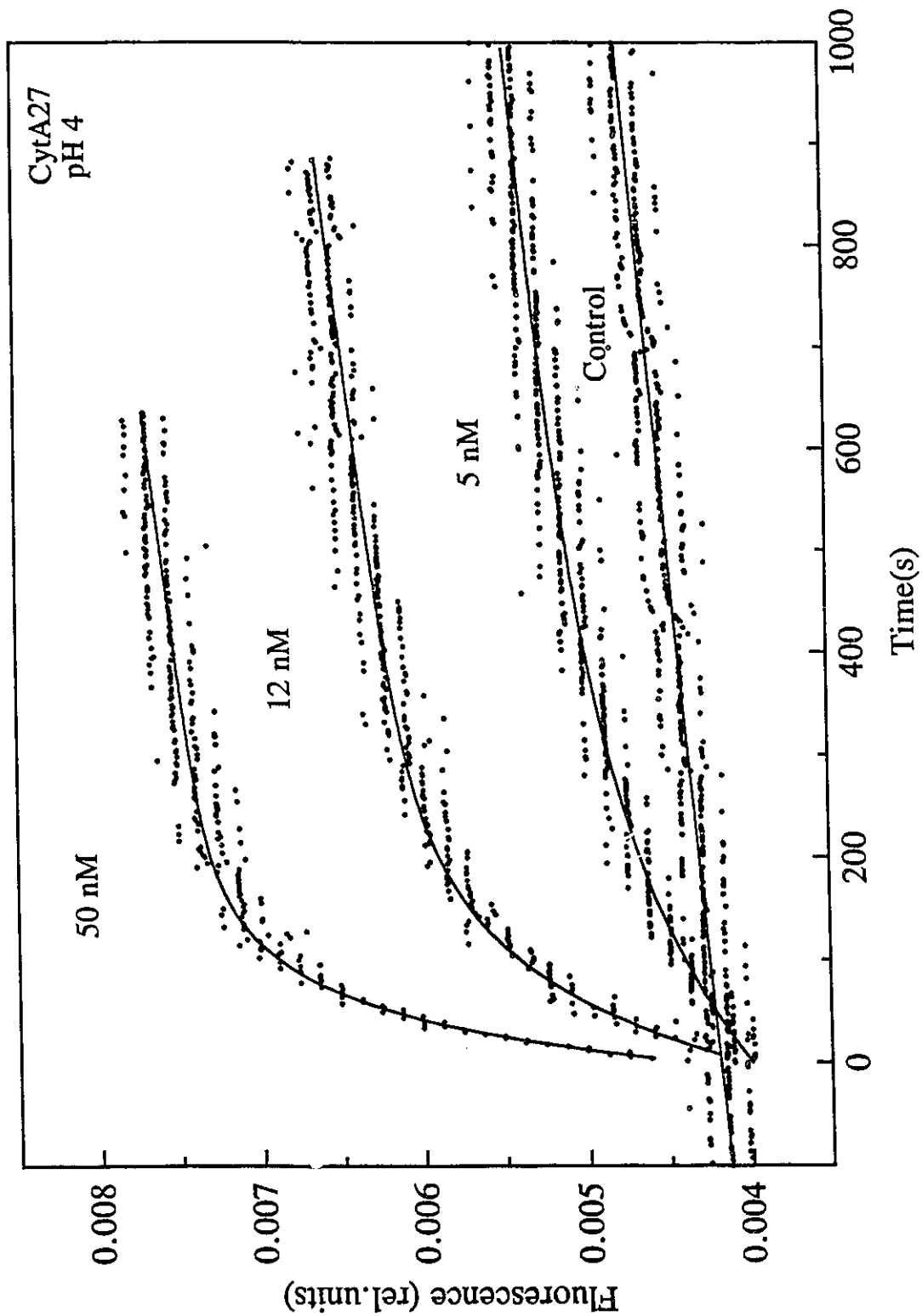


Figure 13. Kinetics of the cytA27 protein induced calcein release from PC-LUV at pH 7.4

5-50 nM Protein were used. The concentration of lipid was 1.2 μ M.

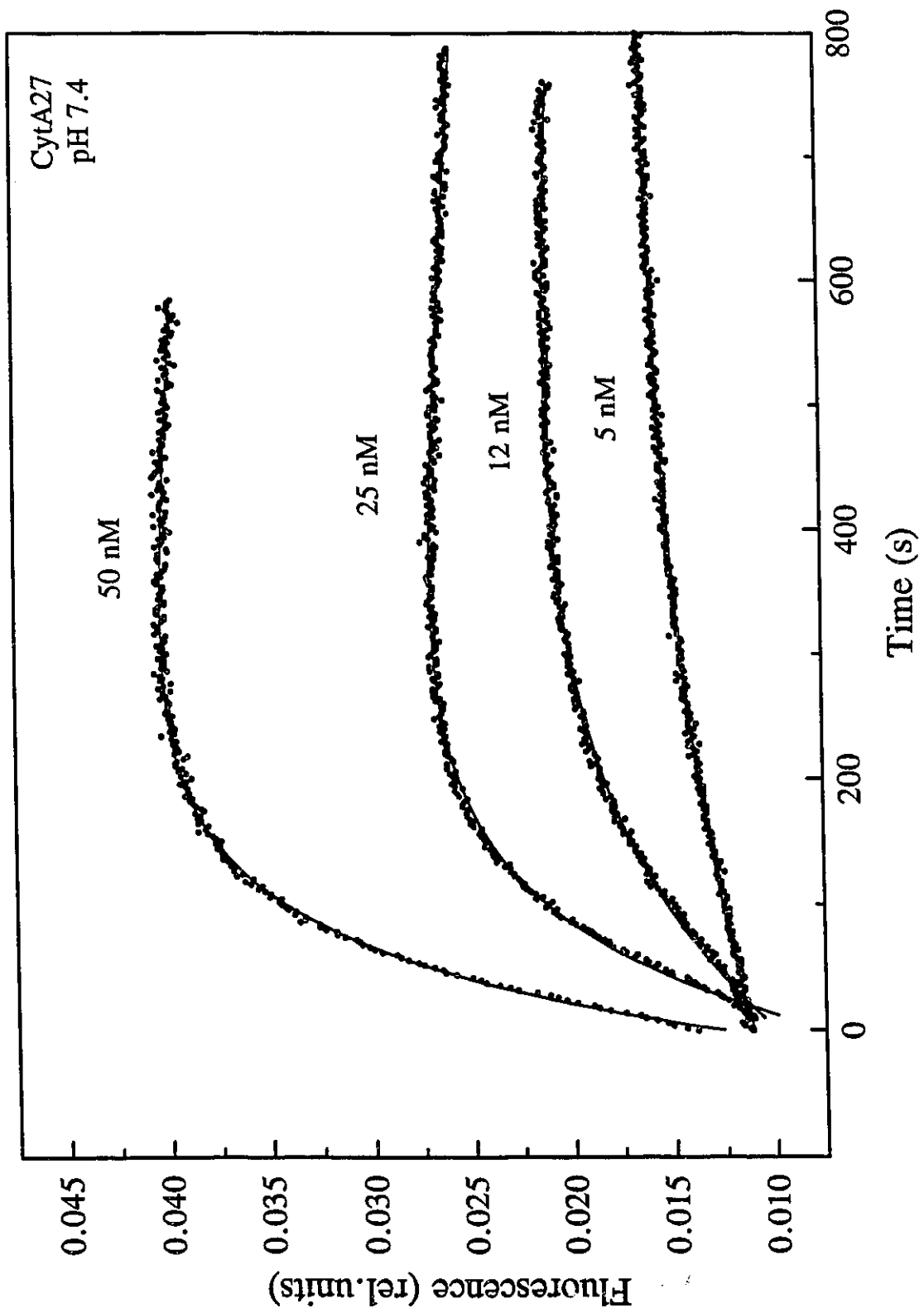
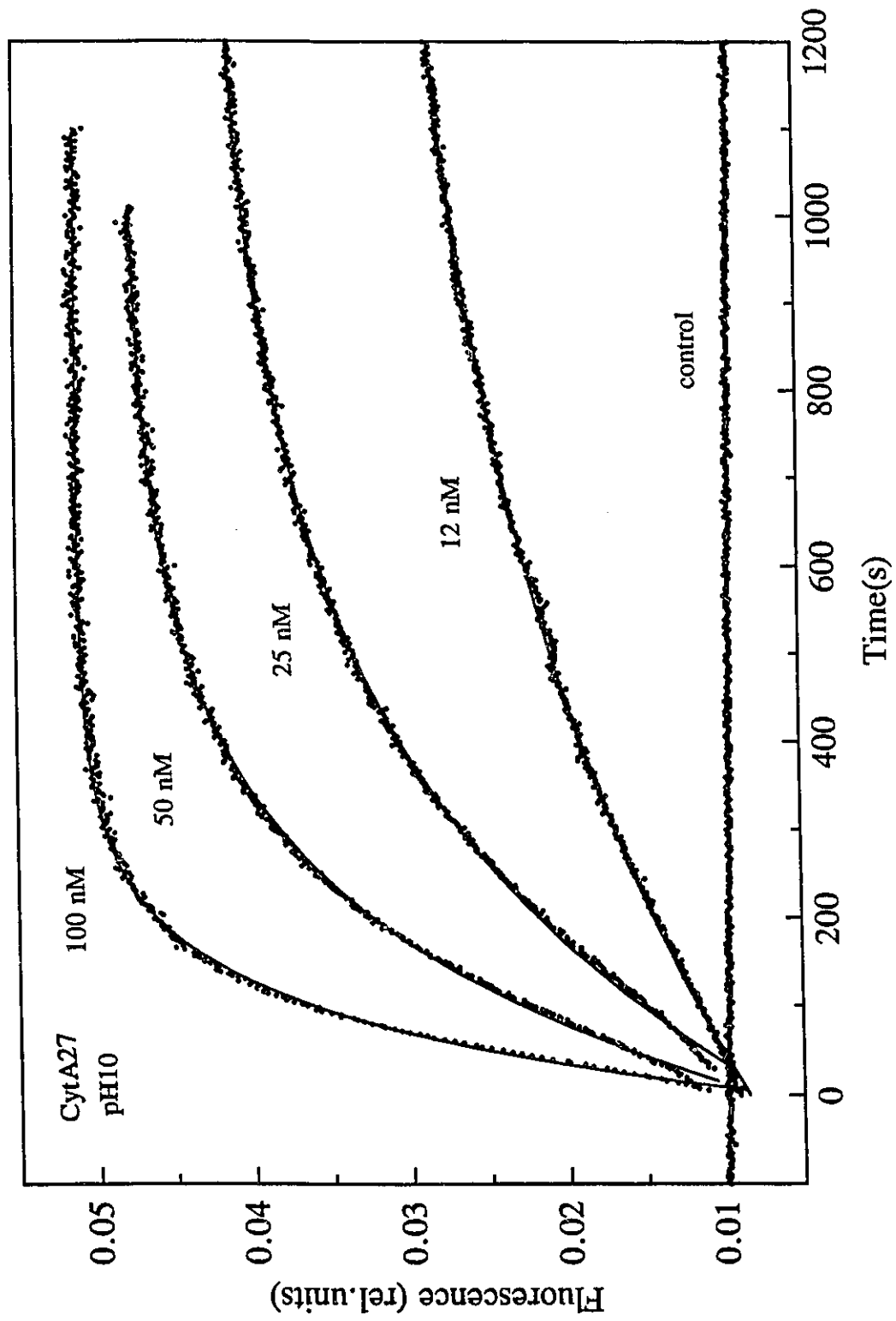


Figure 14. Kinetics of the cytA27 protein induced calcein release from PC-LUV at pH 10.0

The protein concentrations used were in the range from 12 - 100 nM and 1.2 μ M lipid was used.



0
:

Figure 15. Effect of pH on cytA27 induced leakage of entrapped calcein from PC-LUV

Release rate constants at different pH were plotted as a function of protein concentration. The rate constant, k , for each protein/lipid ratio was determined by curve fitting to the kinetic data in Figure 12,13,14.

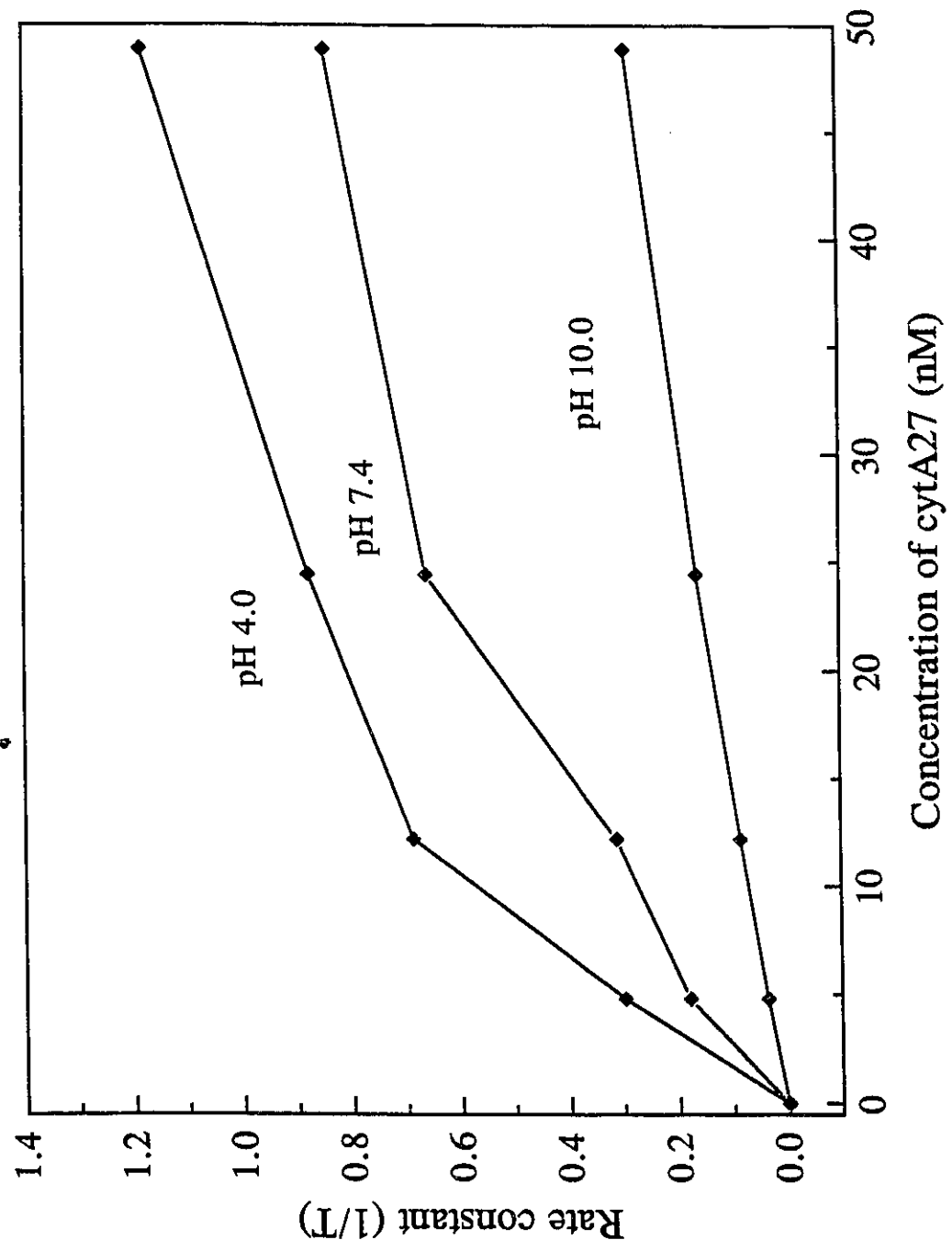


Figure 16. Kinetics of cytA24 induced calcein release from PC-LUV at pH 7.4

1.2 μM lipid and 5 to 50 nM of the cytA24 protein were used.

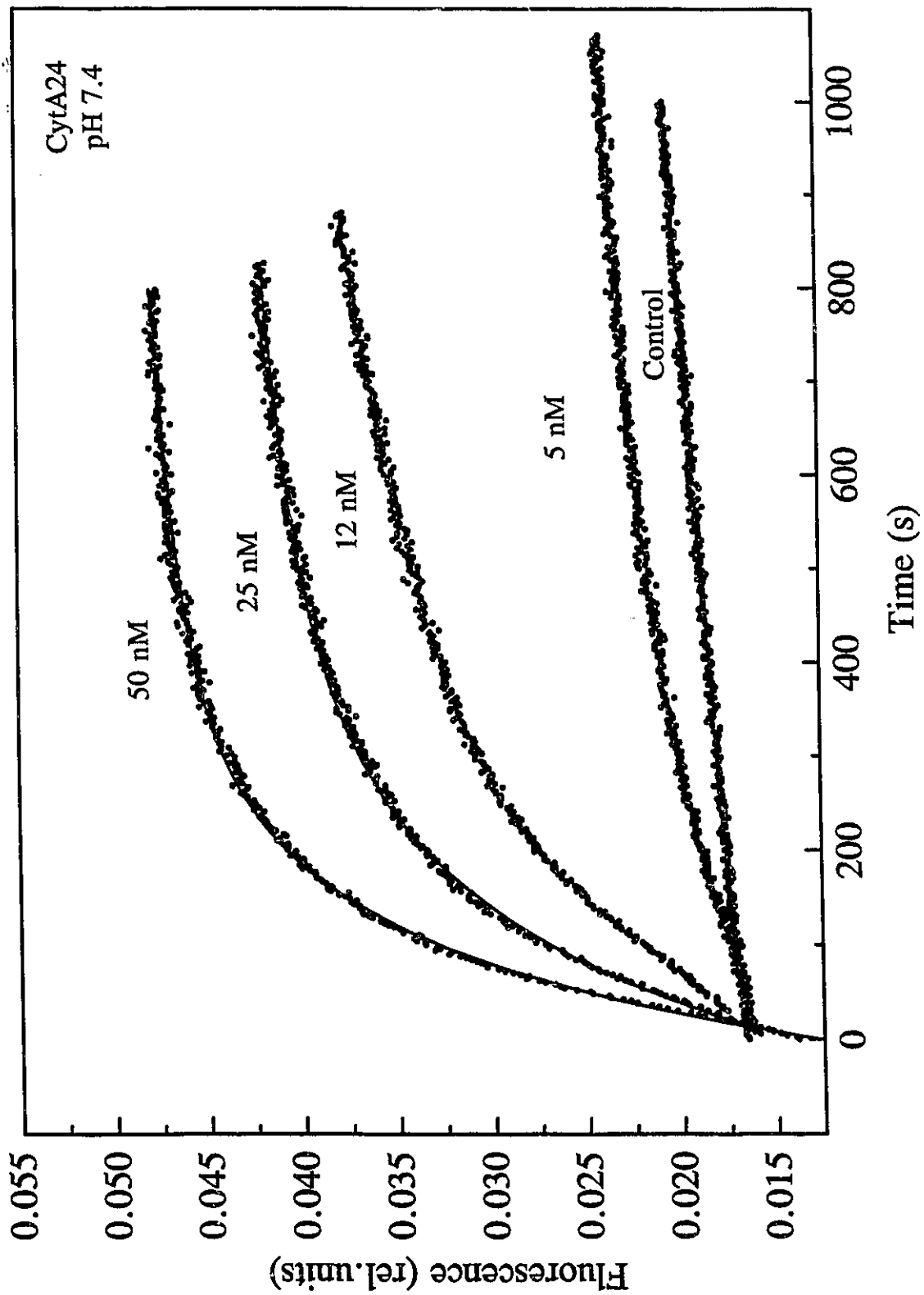


Table 3. Rate constants (min^{-1}) of cytA27 and cytA24 induced calcein release as a function of protein concentration and pH.

Proteins and buffer pH	cytA27 pH 4.0	cytA27 pH 10.0	cytA27 pH 7.4	cytA24 pH 7.4
Protein concentration (nM)				
5	0.298	0.0360	0.178	0.145
12	0.688	0.0852	0.312	0.278
25	0.878	0.163	0.661	0.423
50	1.183	0.288	0.845	0.528
100	-	0.671	-	-

saturated value is reached. This is true for both cytA24 and cytA27, while as seen in Figure 17, at the same protein-lipid ratio, cytA24 always produces a higher extent of calcein leakage. Similar results were obtained with SUV (Figure 18). However, in general, it can be seen that the cytA proteins induce less calcein release from SUV than from LUV.

Figure 19 plots the extent of cytA24 induced FITC-dextran release from LUV at different protein concentrations. The data in Figure 19 are comparable with the curves for the cytA proteins-induced calcein release from LUV and, thus, the molecular weights of the entrapped fluorescence markers do not appear to influence the release properties.

5. Release mechanism:

In the release experiments, 100% of release of entrapped material by the cytA proteins is not reached (Figure 11). There are two models to explain this result: all-or-none release or graded release. For the all-or-none model, some of the vesicles undergo major disruption and release all of their contents, the rest are not disrupted and do not release anything at all. After incubating with the cytA proteins, the interior calcein concentrations of the remaining intact vesicles will be the same as those without treatment by proteins. So the fluorescence ratios (before and after the addition of Triton) of the remaining intact vesicles

Figure 18. The extent of release of cytA27 and cytA24 induced calcein leakage from egg PC-SUV vesicles at a variety of protein and lipid ratios.

Lipid concentrations were 0.6, 3 and 12 μM . Protein concentrations were from 12 to 180 nM. CytA24 (O--O), cytA27 (\blacklozenge -- \blacklozenge).

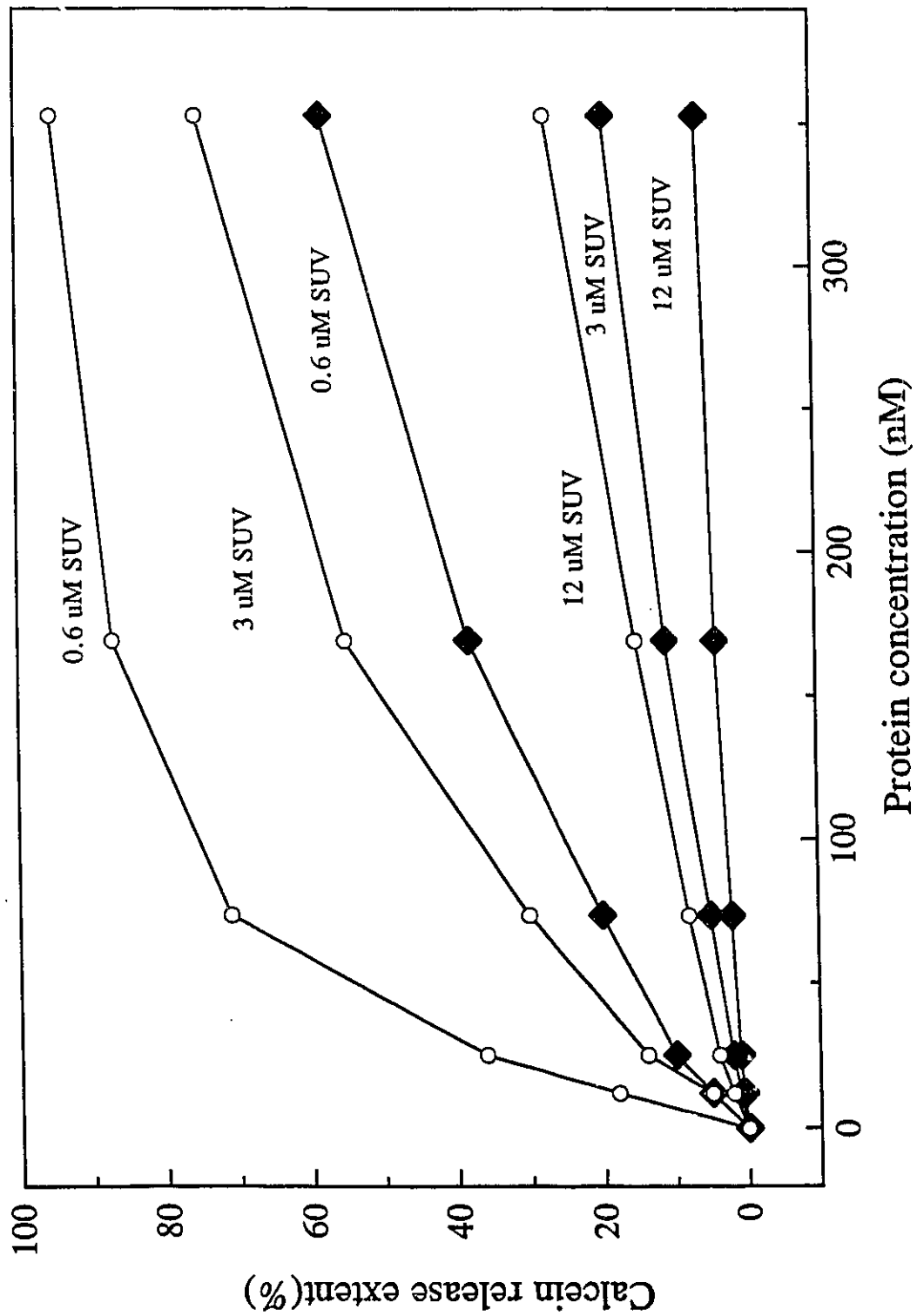
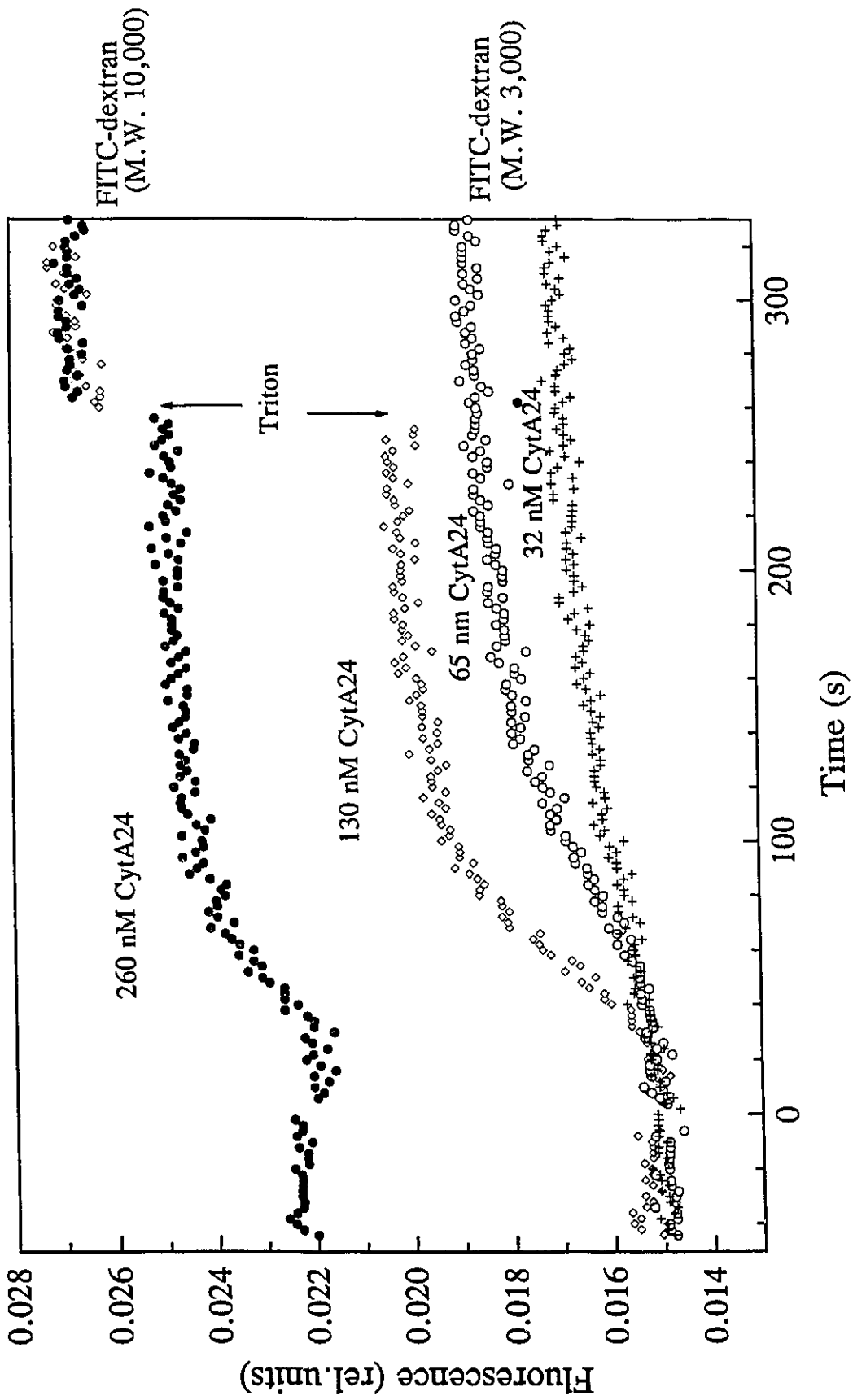


Figure 19. The cytA24 protein induced FITC-dextran release from LUV

●--●: FITC-dextran of molecular weight 10,000, protein concentration 260 nM. ◇--◇, ○--○,
+--+ : FITC-dextran with molecular weight 3,000, protein concentration at 130, 65, 32 nM
respectively.



under several different protein-lipid ratios will be the same and consistent with the values obtained in the absence of proteins.

In the case of graded release mechanism, all of the vesicles release a certain amount of their calcein but remain essentially intact. The interior calcein concentration of the vesicles will change according to the protein and lipid ratios. The concentrations of interior calcein will decrease with increasing protein-lipid ratios. The lower calcein concentration of the vesicles would result in higher fluorescence ratios (ratio of fluorescence signals before and after addition of Triton).

These two mechanisms can be distinguished by the method of Weinstein (1984) discussed in Methods and Materials. The dashed lines in Figure 20 are the predicted value of fluorescence ratios for the all-or-none model. The fluorescence ratios at several different protein-lipid ratios are the same as the values obtained in the absence of proteins. Figure 21 is the standard quenching curve constructed to establish the relationship between interior calcein concentration and fluorescence ratios (the ratio of the fluorescence signal before and after the addition of Triton). This curve was used to predict the fluorescence ratio values for the graded release model. The solid lines in Figure 20 present the predicted fluorescence ratios in the case of graded release.

Fluorescence ratios were measured and calculated as described in Materials and

A

B

Figure 20. Experiments on the mechanism of the cytA proteins induced vesicle leakage.

A:CytA27. B:CytA24. Solid lines are theoretical values predicated by graded release mechanism. Dotted lines are theoretical values predicated for the all-or-none mechanism. Solid circles are experimental values.

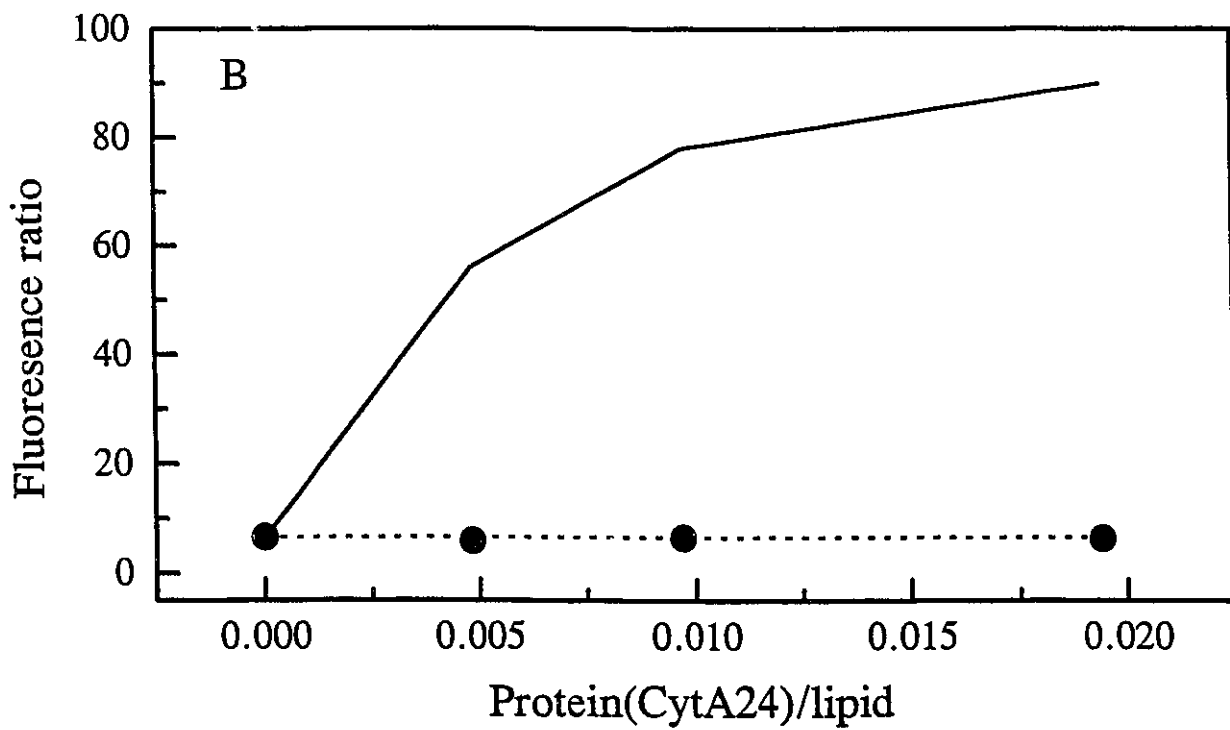
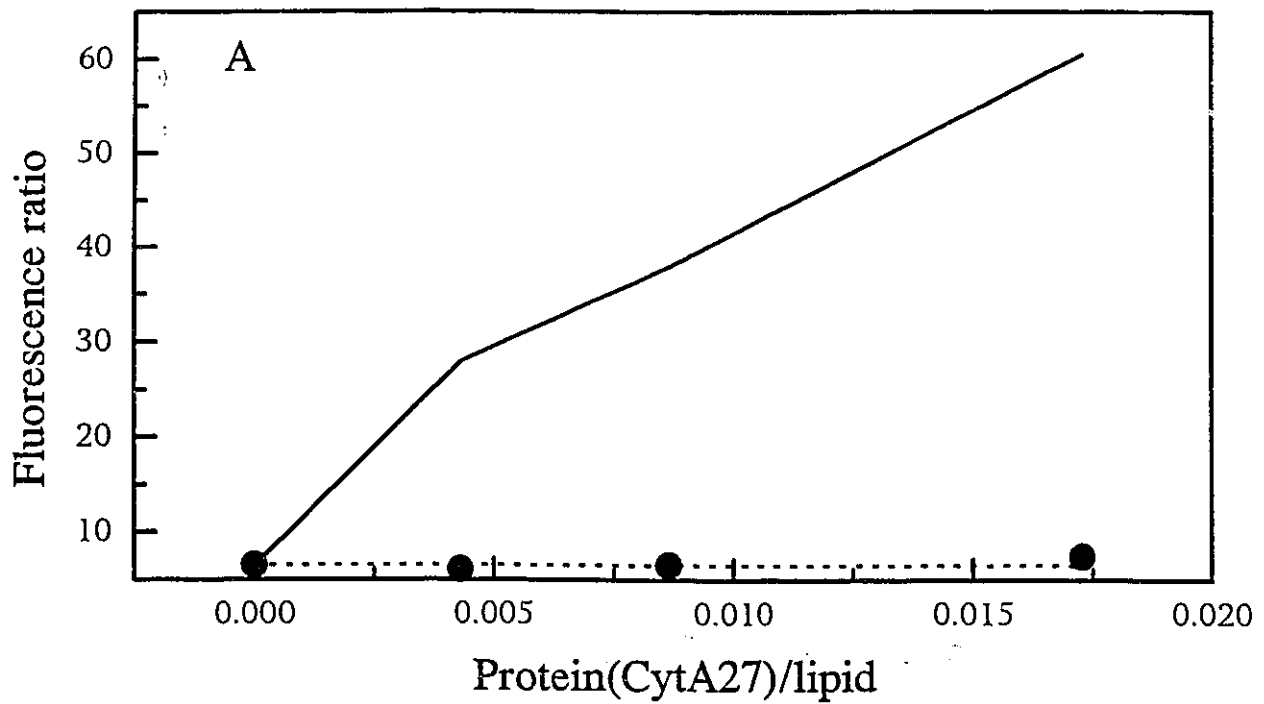
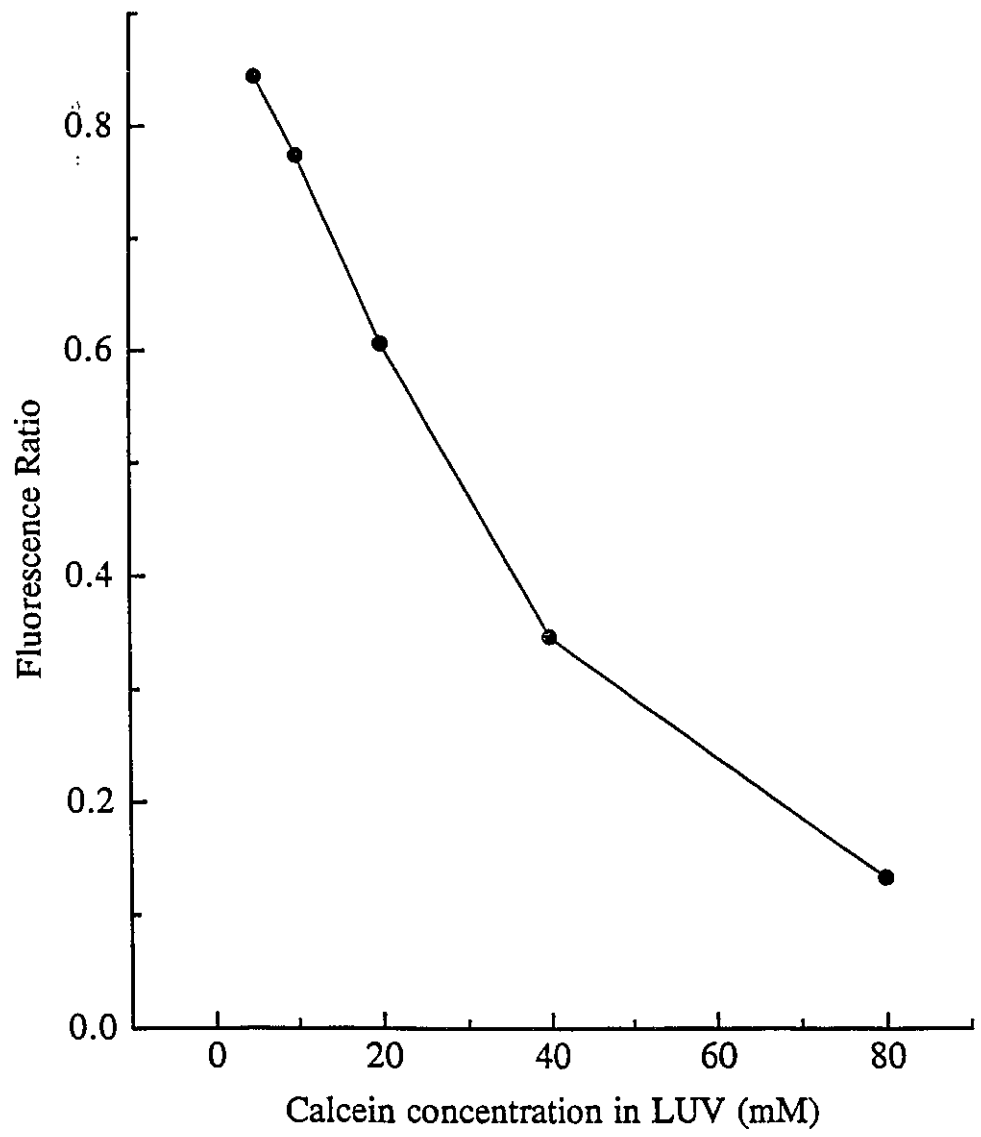


Figure 21. The standard quench curve of the fluorescence quenching as a function of the entrapped calcein concentration in LUV

The pre and post Triton fluorescence levels were determined with vesicles containing 5, 10, 20, 40 and 80 mM calcein.



Methods. Comparison of the observed results and predicted values for both models of release shown in Figure 20 indicate that cytA27 induced calcein release (Figure 20A, solid circle) shows a better fit with the all-or-none model (dashed line). Identical results were obtained with the cytA24 protein (Figure 20B). The results imply that a critical value of cytA protein molecules must be reached for a complete release of calcein from a single vesicle.

6. Determination of cytA protein-lipid binding isotherms:

As discussed in the foregoing section, the calcein leakage from egg PC vesicles is dependent upon the relative concentrations of protein and lipid, which suggests involvement of a binding process in the leakage phenomenon. To investigate further cytA protein-induced leakage, a method to determine the affinity of permeabilizing agents to liposomes was employed (Matsuzaki et al., 1989). The affinity of proteins to vesicles and the amount of membrane-bound protein per vesicle necessary for leakage to occur were estimated by analyzing protein concentration dependency of the leakage at three fixed lipid concentrations as shown in Figure 22 and 23.

As shown in Figure 11, cytA protein-induced calcein leakage can be monitored fluorometrically as a function of time with 100% release being obtained by addition of Triton X-100. The present experiments were carried out

at a number of protein and lipid ratios. The rate constants for fractional calcein leakage, k' , were obtained based on these data using the equation $k' = \Delta F / (F_{100} \times t)$; with k' being expressed as % leakage per minute. ΔF is the fluorescence intensity change at a certain period of time t at each protein and lipid ratio. F_{100} is the fluorescence intensity after adding Triton. From Figures 22 and 23, by drawing a set of lines parallel to the x axis through the dose response curves, several pairs of P_0 (the total protein concentration) and L (the lipid concentration) values at an equivalent leakage rate were obtained. A P_0 and L plot was constructed based on these sets of values for both the cytA27 and cytA24 proteins and is shown in Figure 24. The leakage rate is assumed to be determined only by the amount of membrane-bound protein per lipid, r . The value of r is related to P_0 and L through a conservation equation:

$$[P]_0 = [P]_f + [L]r$$

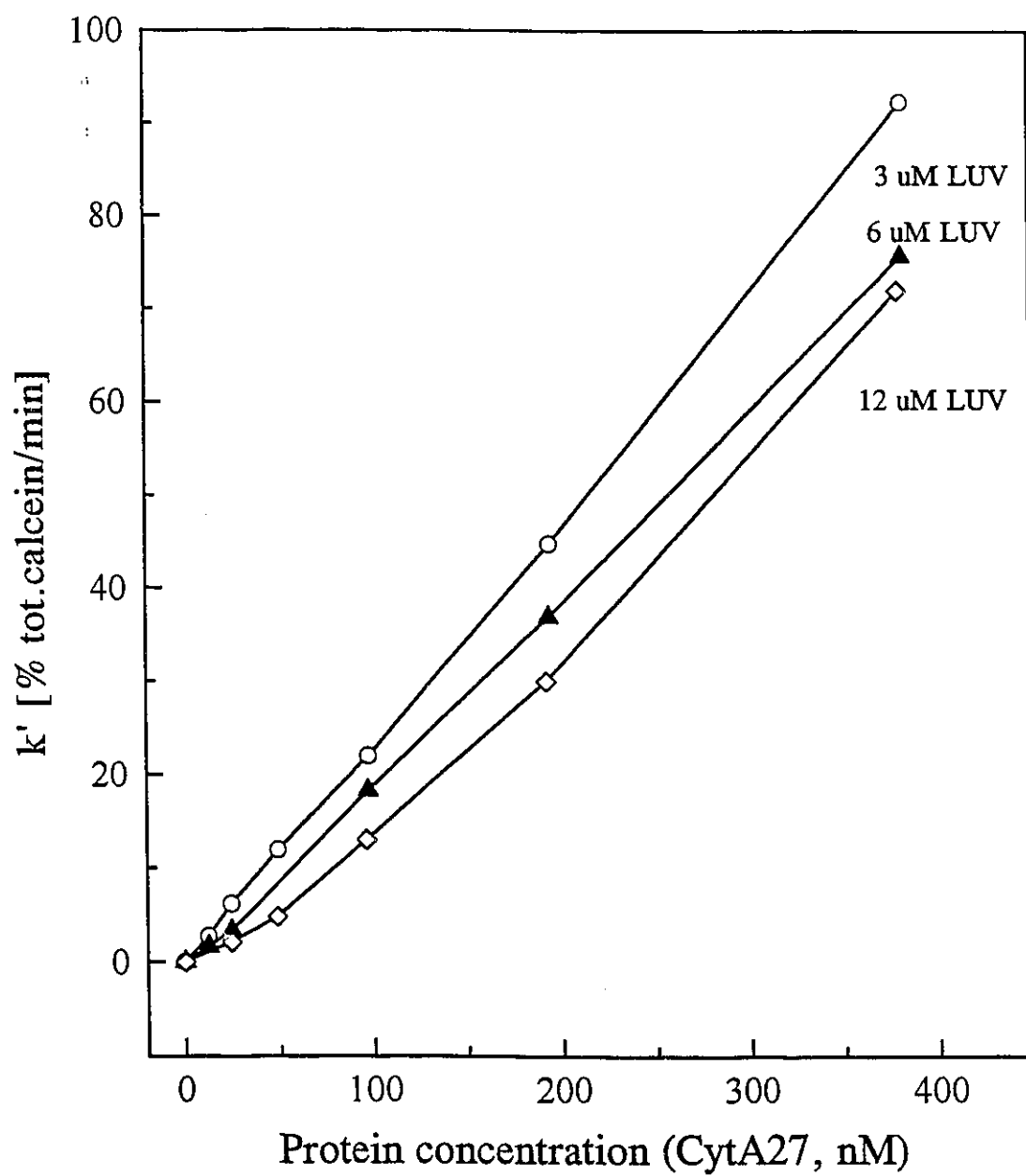
Where $[P]_f$ stands for the free protein concentration. Since all pairs of P_0 and L values that produce an equivalent leakage rate will also have the same value of r , a P_0 versus L plot at any given leakage rate will give a linear relation (Figure 24), and values of r and P_f can be estimated from the slope and intercept of each line, respectively.

Binding isotherms of the cytA27 and cytA24 proteins with lipid were then obtained from a plot of $[P]_f$ against r values as shown in Figure 25. An

apparent binding constant k_a (the ratio of bound to free protein per unit of lipid) was obtained from the slope of each line with the two proteins giving values of $k_{a,\text{cytA27}} = (1.84 \pm 0.11) \times 10^4 \text{ M}^{-1}$ and $k_{a,\text{cytA24}} = (8.33 \pm 0.55) \times 10^4 \text{ M}^{-1}$. Since only the outer monolayer of the vesicles (54% of total lipid) would bind, the k_a values were modified as, $k'_a = k_a/0.54$. $k'_{a,\text{cytA27}} = (0.34 \pm 0.02) \times 10^4 \text{ M}^{-1}$ and $k'_{a,\text{cytA24}} = (1.54 \pm 0.10) \times 10^5 \text{ M}^{-1}$. In Figure 26, the leakage rates are plotted as a function of the amount of bound protein r . Therefore the minimal (when leakage commences) amount of membrane-bound protein needed to induce leakage can be estimated from the intersection on the x -axis. They are 0.0039 ± 0.00019 proteins/lipid for cytA27 and 0.0019 ± 0.00027 proteins/lipid for cytA24. These numbers can be expressed as 256 ± 11 lipid/protein (cytA27) and 526 ± 51 lipid/protein (cytA24), which is the molecular ratio of lipid to protein when leakage commences. Significant differences are observed between cytA24 and cytA27 proteins with the r value of cytA27 being almost two fold higher than that of cytA24, suggesting that more molecules of cytA27 are required to bind to each lipid vesicle to induce leakage than for the cytA24 protein. If the average outer diameter of a LUV is assumed to be 100 nm, the membrane thickness to be 4 nm and surface area of each lipid molecule to be 0.7 nm^2 , each LUV will consist of approximately 83,000 lipid molecules. The number of protein molecules binding to each LUV required to initiate leakage

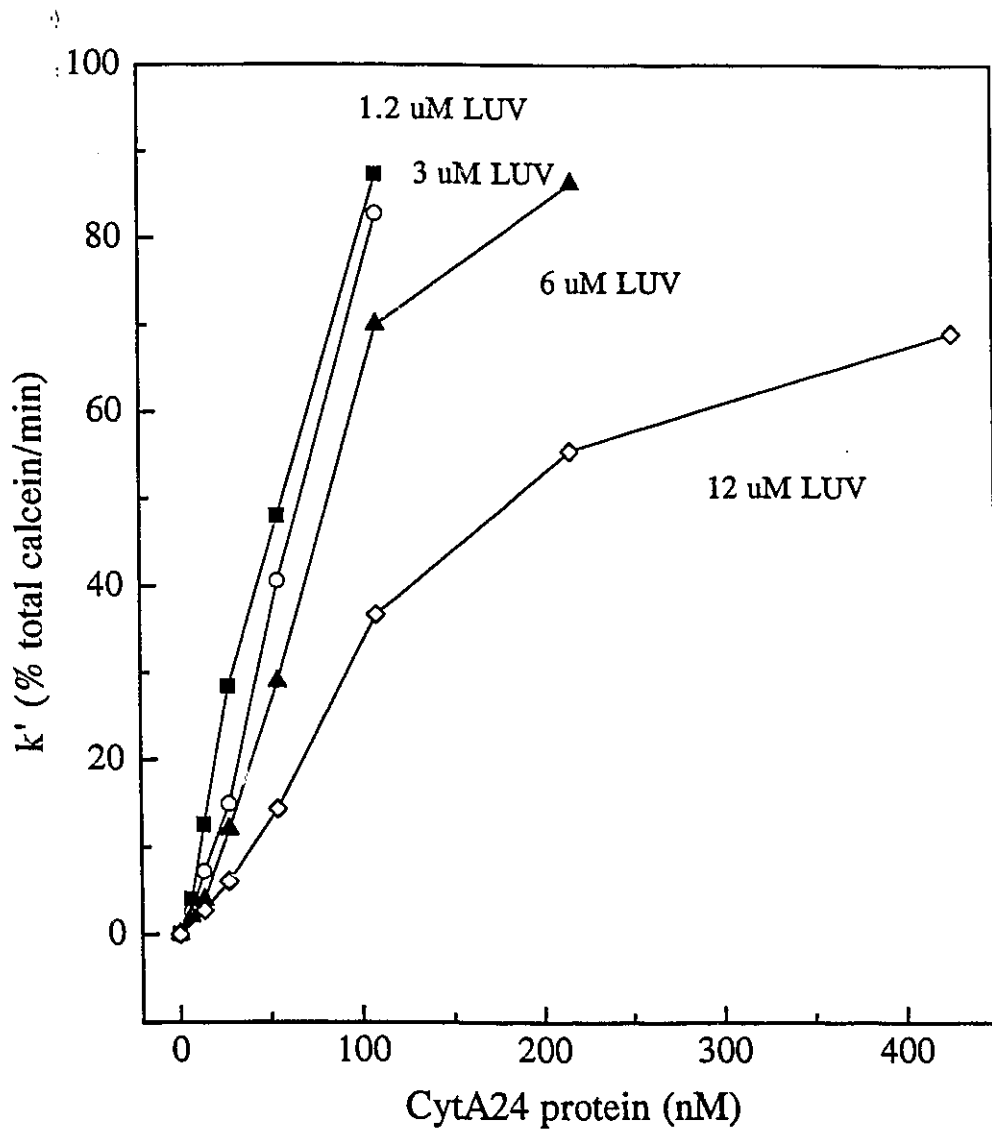
Figure 22. Dependency of calcein leakage rates on the cytA27 protein concentration at three different lipid concentrations

The leakage rate k' is defined as the percentage of calcein release per minute.



4
:

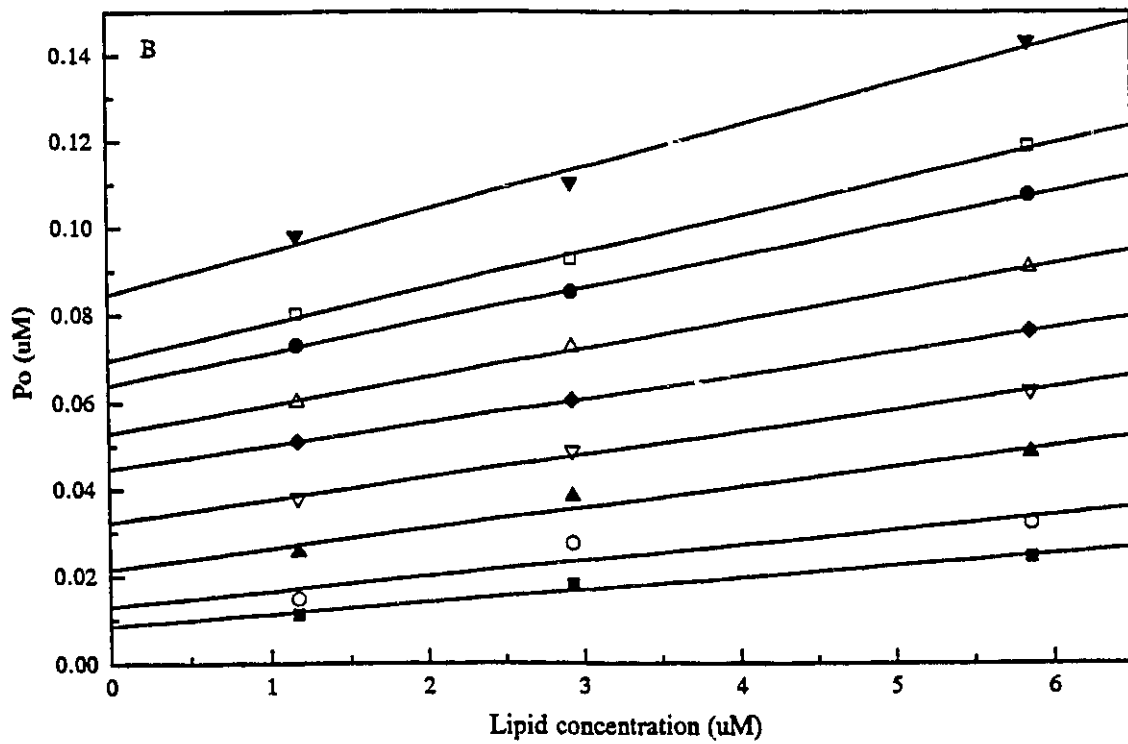
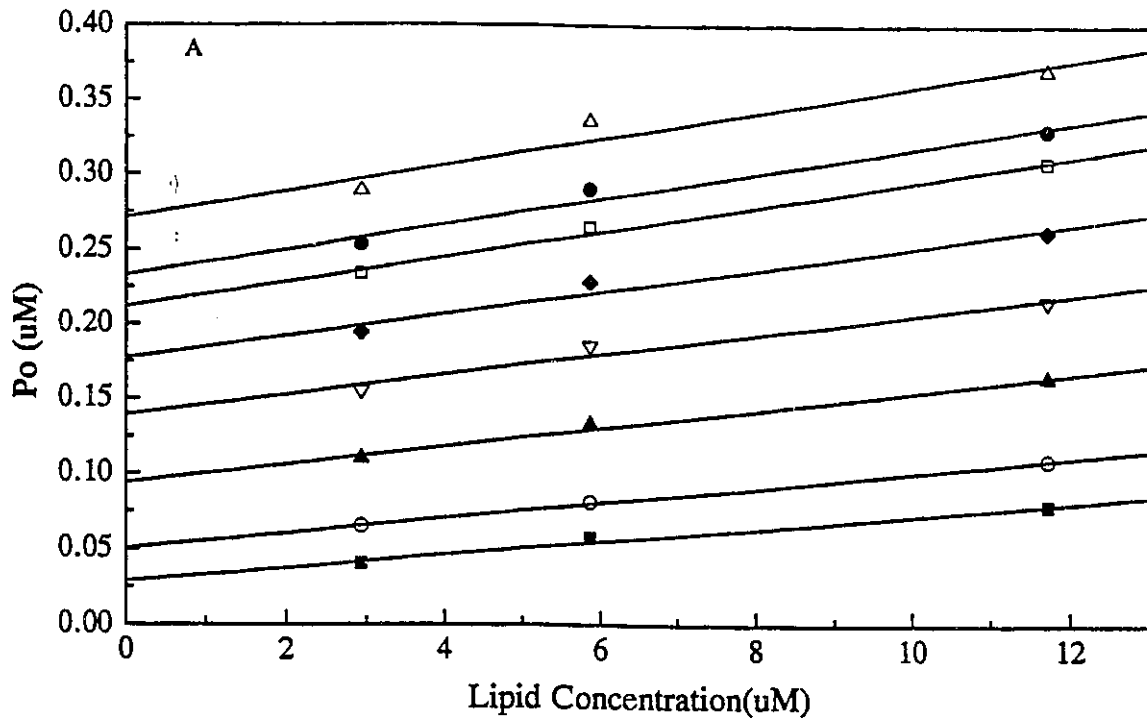
Figure 23. Leakage rate of calcein release as a function of the cytA24 protein concentration at four different lipid concentrations.



9
:

Figure 24. Estimation of free and membrane-bound protein concentrations at several leakage rates.

$[P]_0$ was plotted versus $[L]$ according to the equation in the text. A:CytA27. B:CytA24.



)

:

Figure 25. Quantitative binding isotherms for the interaction of the cytA proteins with PC LUV vesicles.

The pairs of r and $[P]_f$ values were obtained from Figure 24.

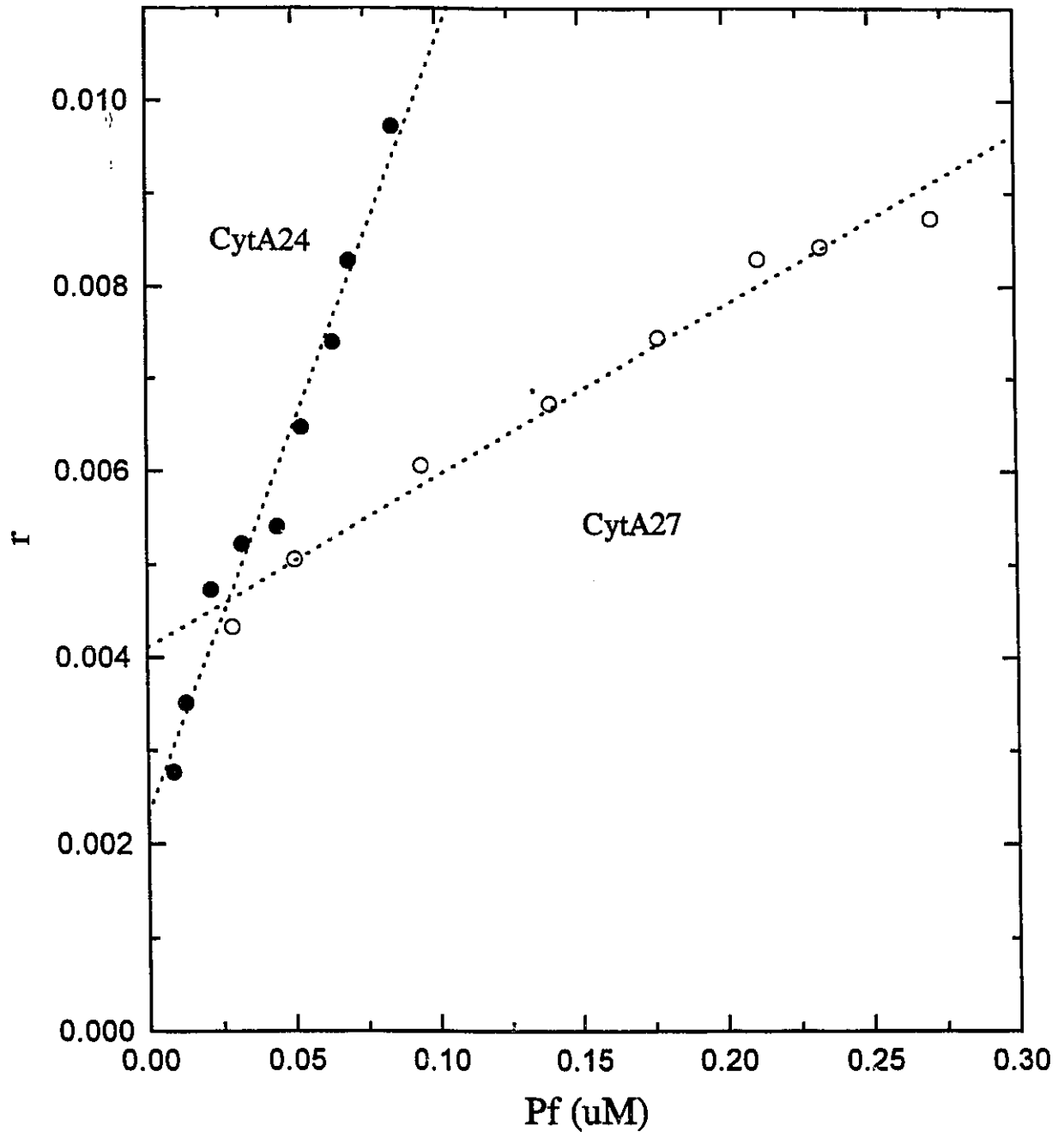
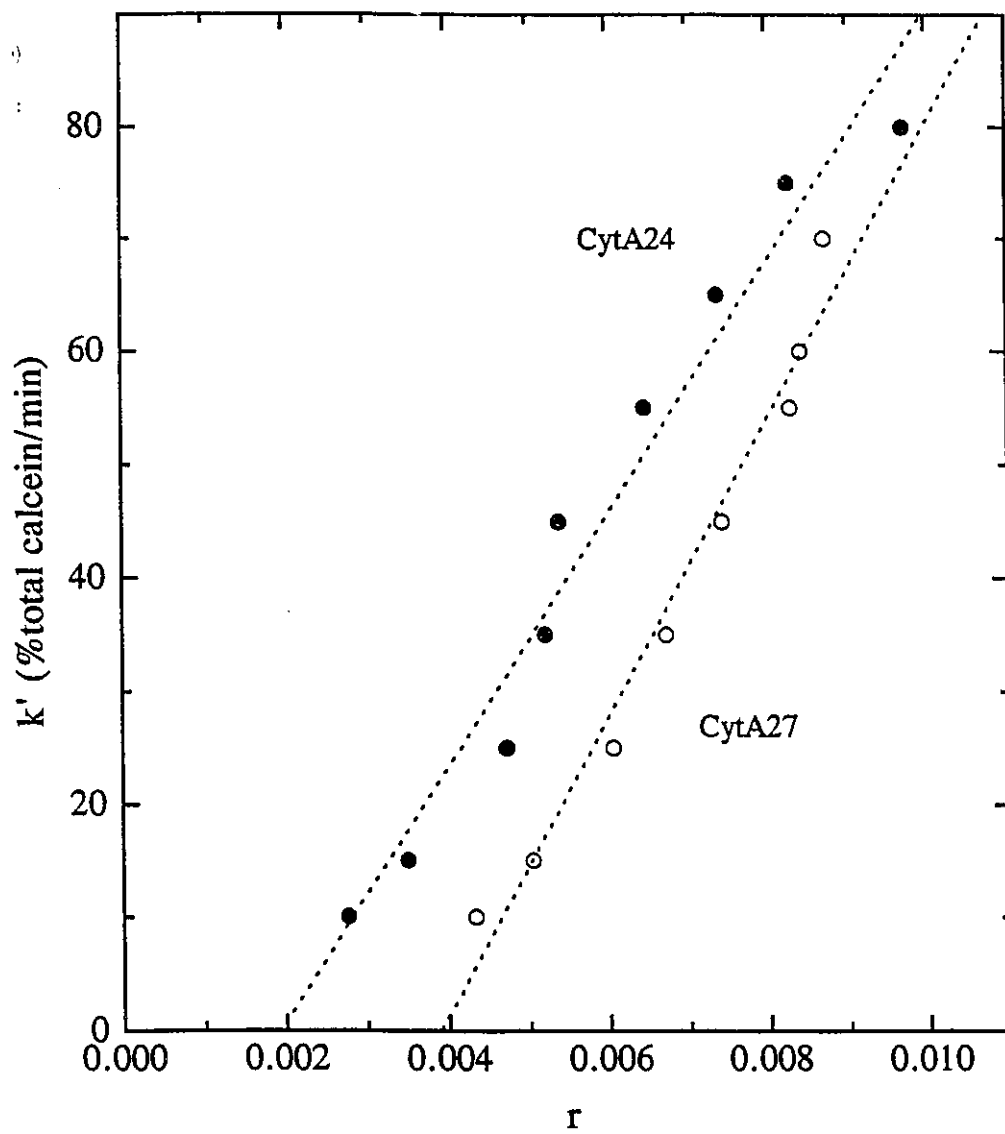


Figure 26. Dose response curves relating leakage rate to the molar ratio r of bound cytA to lipid.

r and leakage rates were obtained from Figure 24.



can be calculated from the data as 157 ± 22 cytA24/LUV and 324 ± 15 cytA27/LUV.

7. Quenching of the tryptophan fluorescence of cytA27 and cytA24 proteins

Fluorescence quenching is broadly defined as a decrease in the fluorescence intensity of a given substance. Since the quenching process requires close approach between the fluorophore and quencher, it has been widely used in protein studies to estimate the fraction of surface localized tryptophan residues, or permeation of proteins by quenchers.

Two kinds of tryptophan fluorescence quenchers, a neutral quencher acrylamide, and an anionic quencher iodide, were employed in our study of cytA binding to lipid vesicles. Both quenchers are water soluble; acrylamide is larger in size and being electrically neutral, more easily penetrates into protein molecules compared to iodide, which although smaller, is negatively charged and does not easily penetrate into the interior of a protein to bring about quenching. The information available from tryptophan fluorescence quenching in the absence and presence of lipid has the potential to reveal structural features, conformational changes resulting from binding and the relative permeabilities of proteins to quenchers.

For both cytA27 and cytA24, the quenching of tryptophan fluorescence was

examined using KI or acrylamide in the absence and presence of SUV. CytA27 protein contains three tryptophan residues while cytA24 protein only has two tryptophan residues (see Figures 6 and 7 for sequences of the cytA24 and cytA27 proteins). Shown in Figures 27-30 are data for KI or acrylamide quenching. The observed fluorescence decrease results from the quenched fraction of tryptophan residues. Non-quenched or partially quenched fractions contribute to the remaining fluorescence. Quenching of more than one population of tryptophan fluorescence can be analyzed and fitted to a modified form of the Stern-Volmer equation (Lakowicz, 1983):

$$F/F_0 = f_1/(1+K_1[Q]) + f_2/(1+K_2[Q])...$$

Where F is the tryptophan fluorescence at quencher concentration [Q]. F₀ is fluorescence in the absence of quencher. The fraction of the tryptophan (with contribution from 1, 2, 3 tryptophans per protein molecule) having a quenching constant of K_i is f_i. In our work, fits were achieved for a single population f₁ = 1 or two classes of population f₁ and f₂. For acrylamide quenching of cytA24 and cytA27 proteins, a good fit to the data could be achieved using f₁ = 1. However, for fitting the data of KI quenching of both cytA27 and cytA24 tryptophan fluorescence, two f₁ and f₂ values were required. In the fittings, K₂ was fixed at zero since usually it was a small or negative number. In Table 4, the effective quenching constant, K_{eff} = ∑_i f_iK_i, represents the relative

quenching effectiveness. Higher K_{eff} indicates more effective quenching with less quencher required to achieve a certain decrease of fluorescence. The parameters used to fit the quenching data are summarized in Table 4. K_{eff} in the absence of lipid (SUV) for both acrylamide and KI quenching of cytA24, cytA27 proteins is almost twice the value found in the presence of lipid vesicles. The difference is interpreted as the protective effects of lipid which for the bound proteins make some tryptophan residues inaccessible. No significant differences in K_{eff} were obtained between cytA24 and cytA27 proteins when quenched by acrylamide or KI although they have one tryptophan difference in their amino acid composition. Comparison of KI and acrylamide quenching illustrates that considerably larger amount of quenching is observed for acrylamide compared to KI with the same protein. This result probably indicates that at least one population of the tryptophan is in the interior of protein and is not readily accessible to iodide.

Figure 27. Quenching of the cytA27 protein tryptophan fluorescence by acrylamide in the absence (open circle) and presence of egg PC-SUV (solid circle)

Data were fitted by using the equation in the text and fits were shown as dotted lines.

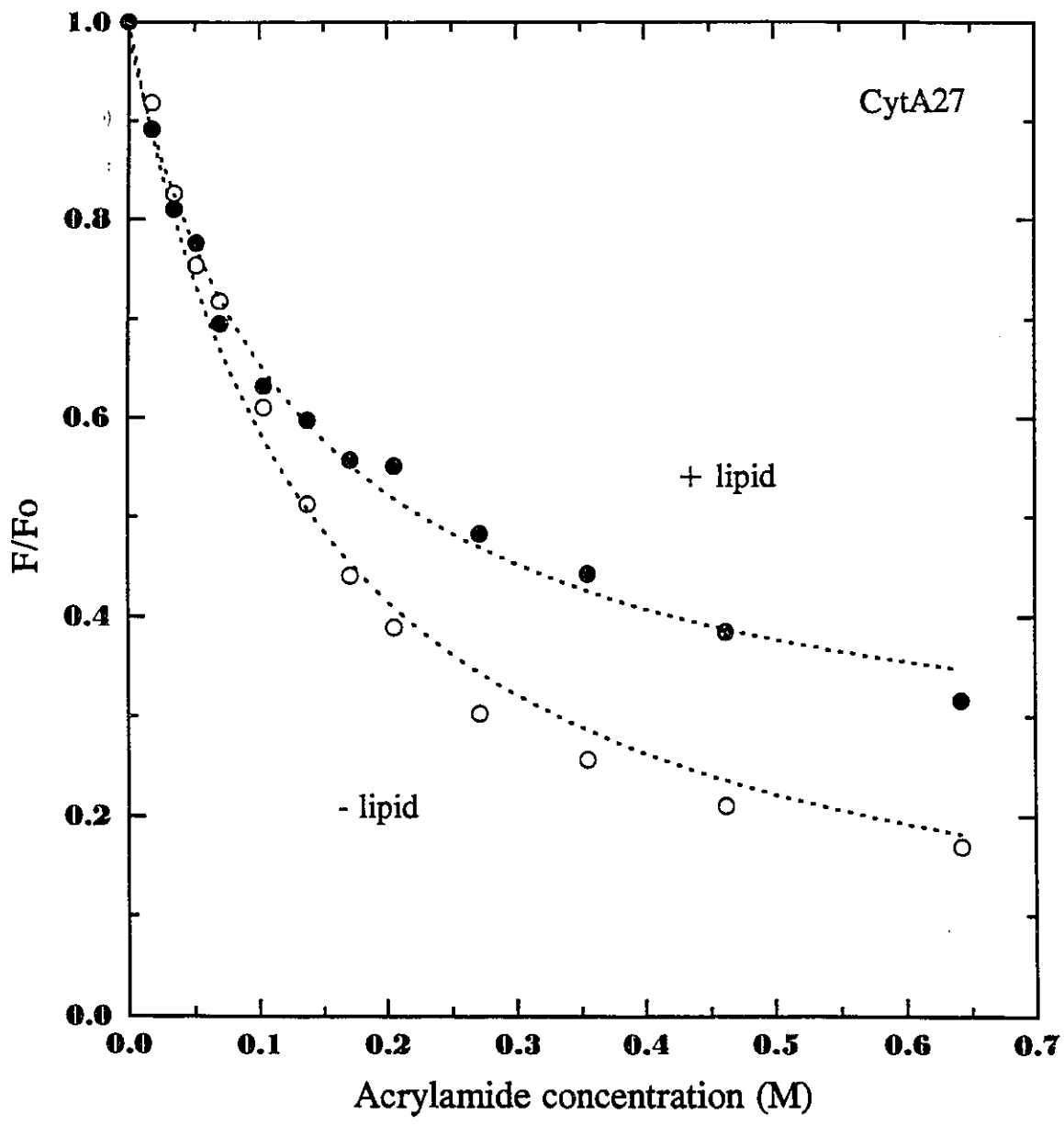


Figure 28. Quenching of the cytA24 protein tryptophan fluorescence by acrylamide in the presence (●) and absence (○) of egg PC-SUV

Data were fitted by using the equation in the text. Quenching parameters, f_i (fraction of tryptophan fluorescence) and K_i (quenching constant) were obtained by fitting the equation to the data.

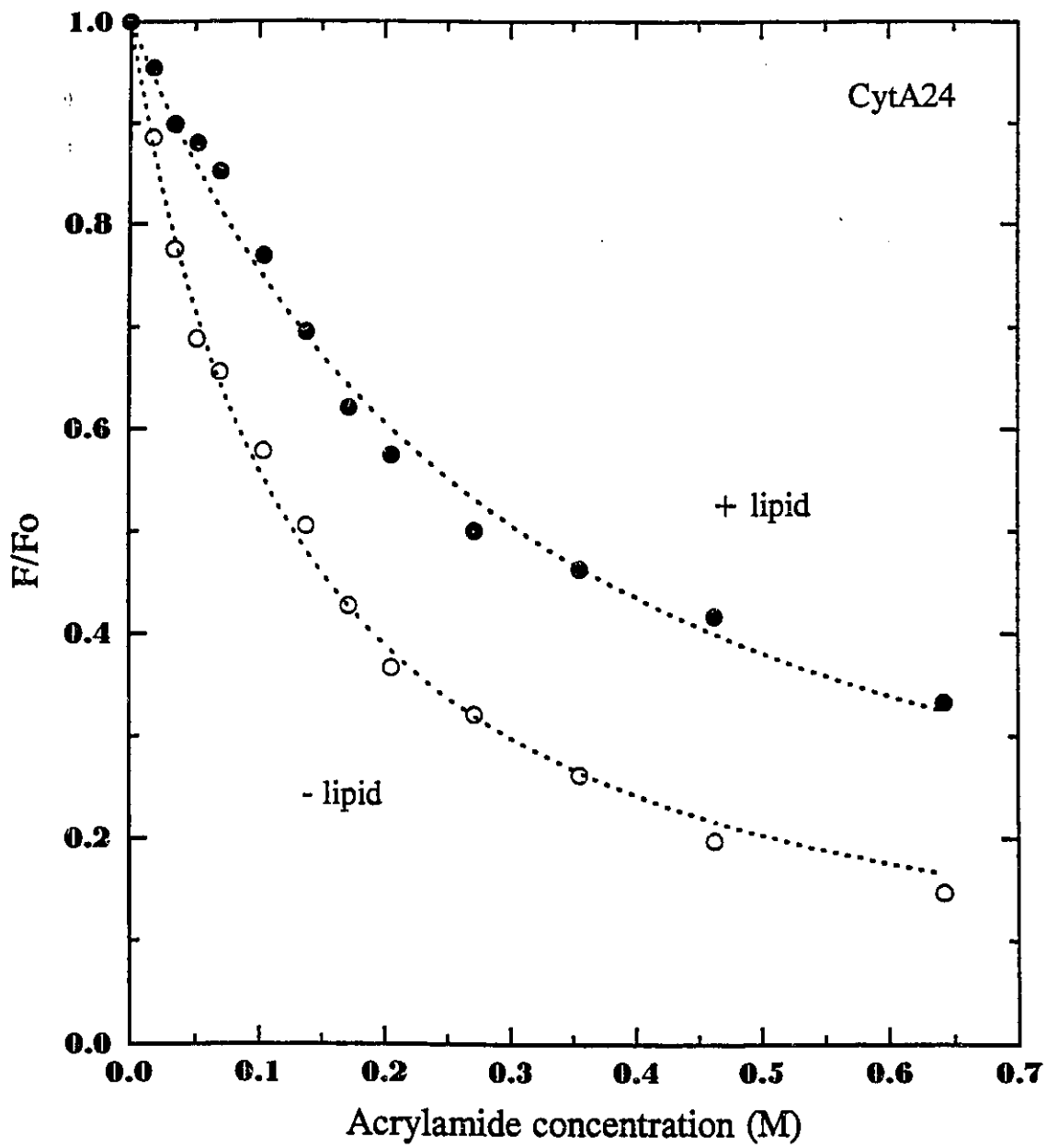


Figure 29. Quenching of the cytA27 protein tryptophan fluorescence by potassium iodide in the absence (open circle) and presence of egg PC-SUV (solid circle).

Data were fitted by using the equation in the text and fits were shown as dotted lines. Quenching parameters, f_i (fraction of tryptophan fluorescence) and K_i (quenching constant) were obtained by fitting the equation to the data.

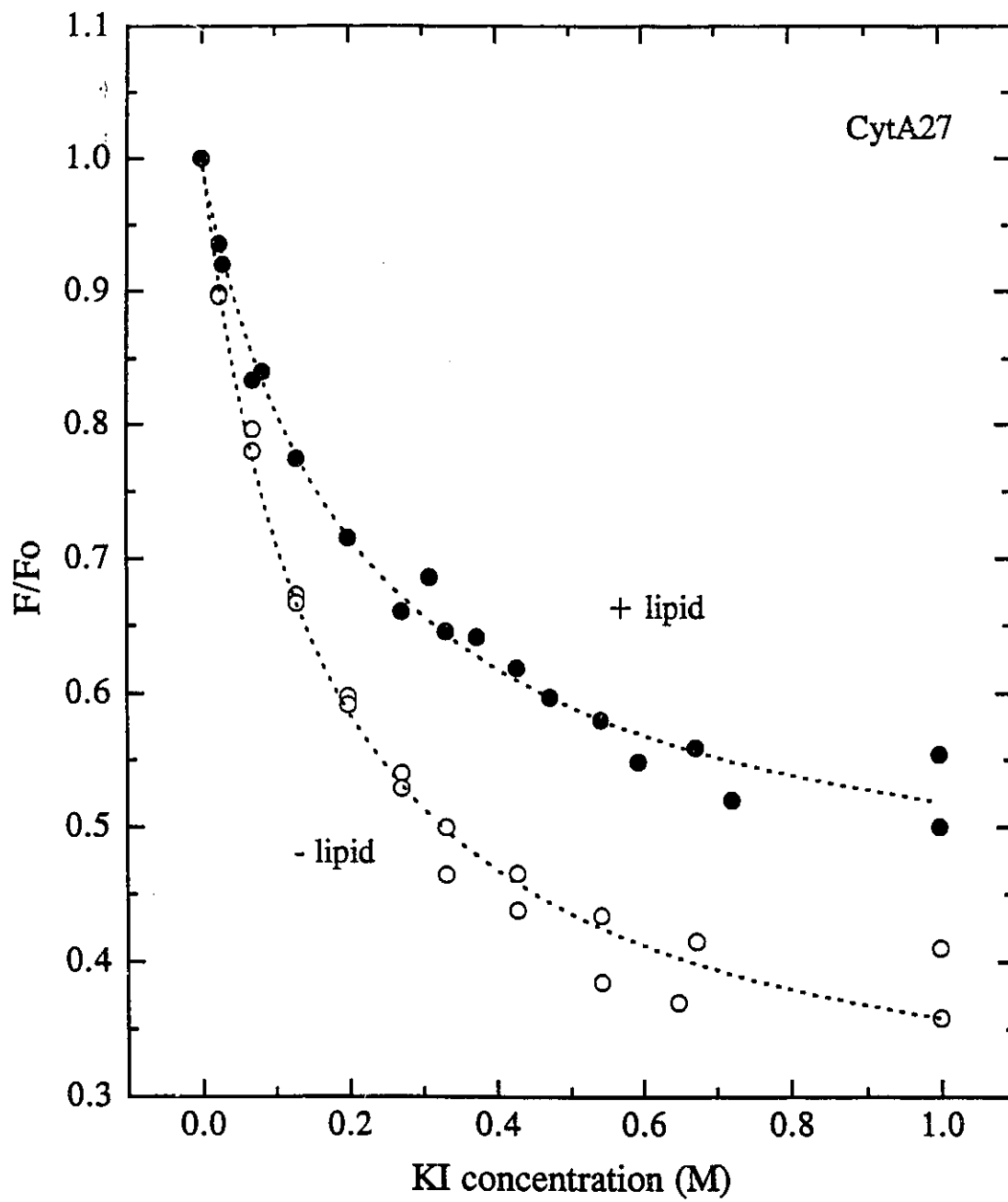


Figure 30. Quenching of the cytA24 protein tryptophan fluorescence by potassium iodide in the absence (open circle) and presence of egg PC-SUV (solid circle)

Data were fitted by using the equation in the text and fits were shown as dotted lines.

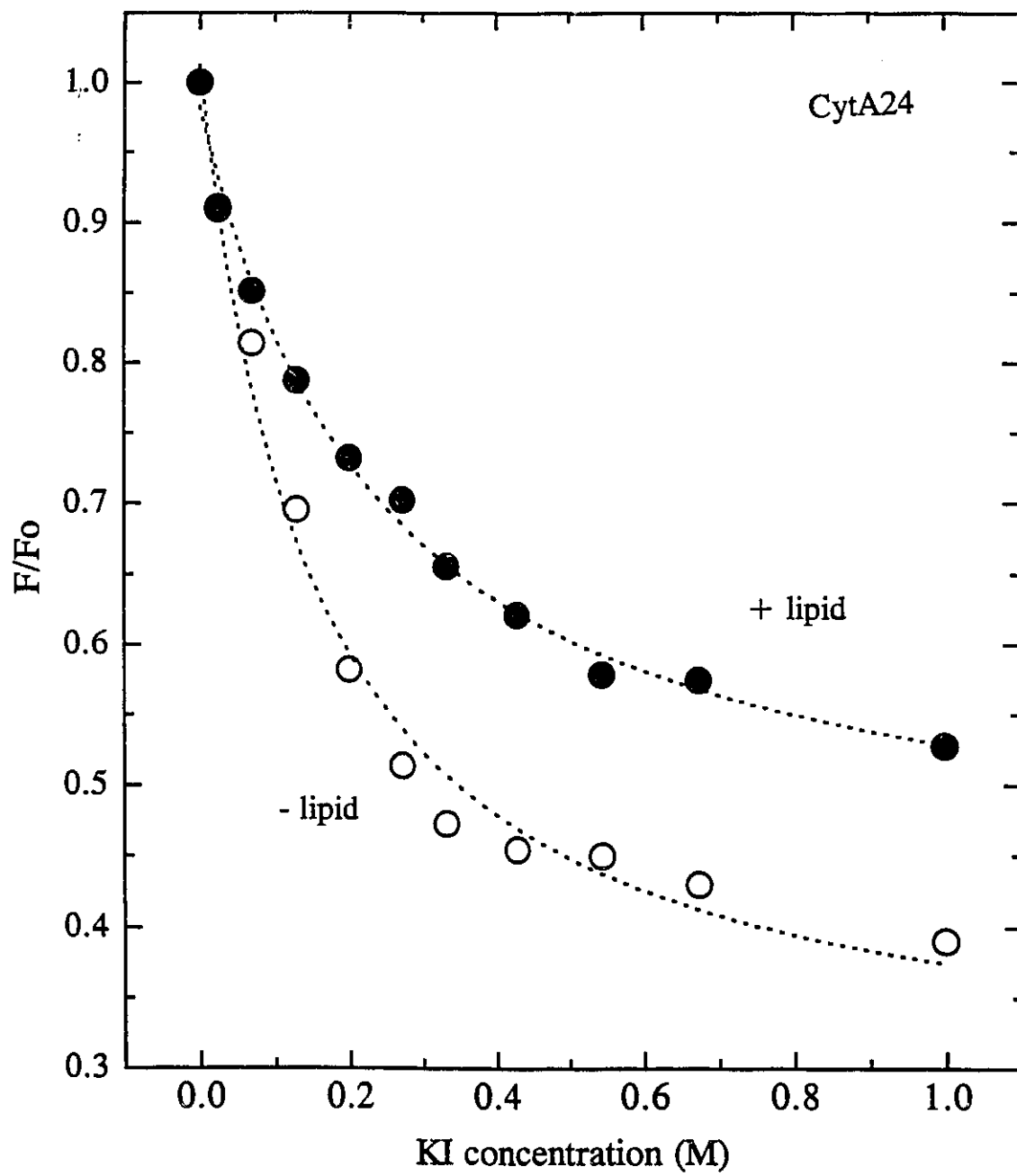


Table 4. Parameters of the cytA27 and cytA24 protein tryptophan fluorescence quenching by acrylamide (AA) and potassium iodide (KI).

Parameters were determined by fitting data of Figure 26, 27, 28, 29 to equation $F/F_0 = f_1/(1+K_1[Q]) + f_2/(1+K_2[Q])$. K_2 was fixed at zero. $f_2 = 1-f_1$. $k_{\text{eff}} = \sum_i f_i k_i$.

Quencher	Protein	Lipid	f_1	K_1	K_{eff}
AA	cytA27	-	1.00	7.0 ± 0.3	7.0 ± 0.3
AA	cytA27	PC-SUV	1.00	4.4 ± 0.3	4.4 ± 0.3
AA	cytA24	-	1.00	7.8 ± 0.2	7.8 ± 0.2
AA	cytA24	PC-SUV	1.00	3.2 ± 0.1	3.2 ± 0.1
KI	cytA27	-	0.75 ± 0.02	6.3 ± 0.6	4.7 ± 0.6
KI	cytA27	PC-SUV	0.58 ± 0.02	4.6 ± 0.5	2.7 ± 0.4
KI	cytA24	-	0.73 ± 0.03	6.6 ± 1.0	4.9 ± 0.9
KI	cytA24	PC-SUV	0.56 ± 0.02	4.1 ± 0.5	2.3 ± 0.4

Discussion

Although, in general, solubilization of Bt subsp. *israelensis* crystals requires alkaline conditions, previous studies have indicated that different conditions are required to dissolve various crystal proteins. In summary, the 27 kDa protein of Bt subsp. *israelensis* was found to dissolve readily above pH 8.3 without addition of a reducing agent; whereas the high molecular weight proteins (135 kDa, 128kDa) could only be solubilized in the pH range of 8.3-9.5 in the presence of a reducing agent. Solubilization of the 65 kDa protein of Bt subsp. *israelensis* requires a pH above 10.5 and a reducing agent (Insell and Fitz-James, 1985, Chestukhina *et al.*, 1985). Basically, our results (Figure 1) are in a good agreement with previous studies except that the solubilization of the 27 kDa protein could only be achieved in buffer pH above 9.5. Moreover, addition of the reducing agent DTT significantly increased the extent of the 27 kDa protein solubilization and achieved better separation from the other crystal proteins giving rise to a higher yield of purified product. Following solubilization, the purification of cytA27 and cytA24 proteins was accomplished by ion-exchange HPLC (see Materials and Methods).

Additional proteins with molecular weights of 30-40 kDa are observed in

SDS-PAGE gels and are considered to be the proteolytic cleavage products of Bt subsp. *israelensis* 130 kDa or 65 kDa proteins (Federici *et al.*, 1990). Also we found that "purified" cytA27 protein if kept in a solubilized form will turn into a 24 kDa protein. This phenomenon is considered to be proteolytic degradation induced by crystal-associated proteases. One group of workers has proposed that these protease activities might be important in the solubilization *in vivo* and biological activity of Bt *israelensis* proteins (Chilcott *et al.*, 1983). The purified cytA24 protein is resistant to further degradation and stable in solubilized form at 4 °C.

For the mass spectrometric technique we used, proteins or peptides normally need to be dissolved at acidic pH (5% acetic acid). Because *B. thuringiensis* proteins are generally only soluble at alkaline pH, it has been very difficult to use mass spectrometry as a tool to determine their molecular weights. Although the crystals of Bt subsp. *israelensis* were initially dissolved at alkaline pH, we were able to dissolve the purified cytA27 and cytA24 proteins in acetic acid and obtain mass spectra. To our knowledge, this is the first example of a *B. thuringiensis* protein molecular weight determination using mass spectrometry. The solubility of cytA proteins at acid pH, which is a property not shared by other proteins of the Bt family, probably originates from their distinctive amino

acid sequences.

The precise molecular basis of the toxicity of *Bacillus thuringiensis* proteins has been the subject of active research. Several models have been proposed to explain the mode of action of Bt proteins. The most widely accepted mode of action is colloid osmotic lysis. It has been suggested that Bt toxins bind to receptors on the membrane and then interact with the membrane to create 'pores' or 'channels'. The generation of those pores leads to colloid-osmotic lysis. Pore formation as a mechanism of cell lysis has been demonstrated to occur for a number of cytolytic toxins, including tetanus (Menestrina *et al.*, 1989), peptide GALA (Parente *et al.*, 1990), and *Staphylococcus aureus* α -toxin (Menestrina, 1986). Recent studies have indicated that several *B. thuringiensis* toxins with different insect specificity act by this mechanism (Drobniewski and Ellar, 1988a; Knowles and Ellar, 1987; Knowles *et al.*, 1989). Another possible mechanism is that Bt proteins may bring about lysis via detergent-like destabilization of the lipid bilayer. Detergent-like destabilization mechanisms are not as widely proposed as pore formation to explain the mode of action of cytolytic toxins, but the former have been put forward to explain the action of several toxins, e.g. melittin (DeGrado *et al.*, 1982; Terwilliger *et al.*, 1982), and streptolysin S (Duncan and Buckingham, 1981).

CytA proteins are cytolytic *in vitro* against a wide range of insect and mammalian cells (Thomas and Ellar, 1983a). They have been shown to interact with lipids and induce permeabilization of phospholipid vesicles (Drobniewski *et al.*, 1987; Drobniewski and Ellar, 1988a). These results suggested that the cytA proteins can exert their cytolytic effects through a protein-lipid interaction, without assistance of the receptors which are utilized in other *B. thuringiensis* toxin-cell interactions (Oddou *et al.*, 1991; Knowles and Ellar, 1986; Knowles *et al.*, 1991). This conclusion is supported by our studies on the kinetics of cytA induced disruption of artificial phospholipid vesicles. Although our results do not rule out the possibility of the existence of a cytA receptor *in vivo*, we show definitively that cytA proteins can exert cytolytic effects in the absence of receptors.

Most published data on the mode of action of cytA proteins support the pore or channel formation hypothesis, indicating that the proteins generate small pores in the membranes, through which small molecules can permeate and give rise to colloid osmotic lysis. Ellar and coworkers observed that cytA proteins cause cell swelling, with small molecules ($^{86}\text{RbCl}$) leaking out before larger ones ($\text{Na}^{51}\text{CrO}_4$) in the case of insect cells. Moreover, osmotic protectants were found to inhibit or delay the cytolysis (Knowles and Ellar, 1987). These

phenomena were interpreted in terms of a pore formation mechanism and the pores were estimated to be 0.5-1.0 nm in radius. In addition, Knowles *et al.* (1989) observed that cytA proteins form cation-selective channels in planar lipid bilayers, which they concluded lends support the pore formation theory and the colloid-osmotic lysis mechanism. Our results obtained from leakage experiments using FITC-dextran (M.W. 3 kDa and 10 kDa), and other data on the release mechanism and on the binding isotherm all appear to favour a detergent-like effect mechanism. If cytA proteins form pores in the membrane, the leakage kinetics will depend on the molecular weights of the entrapped fluorescence substances. However, if the protein acts through a detergent-like manner the leakage kinetics should be independent of the size of the trapped molecule. The results for FITC-derivatised dextran released from LUV support detergent-like action since the release kinetics for dextran from LUV are the same as those for the small molecule calcein (Figure 11). Dextran release is not likely to be a secondary effect of a colloid osmotic disruption since we do not observe a delay or inhibition in the release process of entrapped large molecular weight fluorescence markers.

Our results suggest that the cytA proteins act through an all-or-none mechanism, which has been found for several other lipid vesicle-peptide or

protein systems such as GALA (Parente *et al.*, 1990), magainin 2a (Grant *et al.*, 1992) and α -haemolysin (Ostolaza *et al.*, 1993). The all-or-none lysis of vesicles is also reminiscent of the action of detergents on cells (Scharff and Maupin, 1960; Silhankova, 1959; Nagawa and Regen, 1992). As a result of cytA-induced leakage being an all-or-none mechanism, a critical value of cytA molecules must be reached to induce complete leakage from a vesicle. In turn, this implies that protein aggregation occurs in the membrane, which supports the detergent-like destabilization mechanism.

The binding isotherm experimental data provide further information about the intramembrane protein aggregation. The experiments indicate that extensive intramembrane protein aggregation, with 200-400 proteins per LUVs, on the vesicles is required to initiate the leakage. Studies of the effects of cytA24 protein on the epithelial cells of isolated insect Malpighian tubules suggested that the tubules were unaffected for a relatively long period and then rapid failure occurred (Maddrell *et al.*, 1988), which is consistent with our result from calcein release kinetics experiments where an initial lag period is observed. This additional fact supports the notion that a critical number of protein molecules must accumulate on the vesicle to initiate break down. Taken together these results provide further support that the cytA proteins follow the detergent-

like action mechanism. In a pore formation mechanism, relatively few proteins are required to create a pore or channel and to break down the permeability barrier. From our results and those obtained by others, we suggest that the cytA proteins may resemble other cytolytic toxins such as melittin (DeGrado *et al.*, 1982; Terwilliger *et al.*, 1982) and *streptolysin S* (Duncan and Buckingham, 1981) in their mode of action. The latter are thought to act as a protein surfactant, interacting with membrane lipids directly. This leads to a rearrangement of the lipids such as is caused by detergent, or a partial solubilization of lipid, disrupting the membrane integrity and eventually lysing the cells. However, this does not exclude the possibility that at a certain stage small pores may be formed. We employed a phospholipid vesicle system in our study which is different from the insect cell lines and planar bilayer used by Ellar *et al.*, so our results and the previous studies may not directly be comparable. It is possible that cation-selective channel formation is an additional effect of the cytA proteins. The use of whole cell systems makes it difficult to determine whether the observed effects are induced directly by protein-membrane interactions. The difference in shape between planar phospholipid bilayers and vesicles makes it difficult to compare results from these two systems.

There is no precise definition of 'pores' and 'holes' which may be generated in a membrane through the interaction of protein and lipid. The use of this term in the literature leads to considerable misunderstanding and confusion. These terms do not necessarily connote a well defined, fixed structure in the membrane. 'Pores' can be temporary membrane disruption or a membrane permeability change. From this point of view, our results do not necessarily contradict previous studies.

The amount of membrane-bound protein per lipid molecule, r , reflects the protein's membrane-perturbing ability. In our experiments, r ranges from 0.002 for cytA24 to 0.004 for cytA27. These r values are comparable with the membrane perturbing peptide hypelcin A which has a $r = 0.005$ (Matsuzaki *et al.*, 1989) and one order of magnitude lower than those for magainin 1 ($r = 0.03$) (Matsuzaki *et al.*, 1989) or a detergent Triton ($r = 0.05$) (Paternostre *et al.*, 1988). Thus, the cytA proteins have a higher membrane-perturbing activity compared with those of magainin 1 and Triton.

In most previous studies on the cytA proteins mode of action, the 'activated' form of cytA, cytA24, was used and considered to be responsible for the toxicity and channel forming ability in those studies (Maddrell *et al.*, 1988, Knowles and Ellar, 1987; Knowles *et al.*, 1989). Our results demonstrate that

both cytA27 and cytA24 proteins are able to induce permeability in phospholipid vesicles. However, cytA24 has a higher activity. Fewer cytA24 molecules (157 per LUV) are required to initiate calcein leakage from vesicles compared to cytA27 (324 per LUV). The results suggest that although cytA27 and cytA24 differ by only 30 amino acids, the minor structural change at the N-terminal of cytA27 protein does influence activity.

The fluorescence experiments provide answers to several questions concerning the interaction of cytA proteins with lipid membranes. Figure 15 shows that cytA27 induces calcein release from liposomes in a pH-dependent manner, as the pH increases, the release rate constant decreases. A pH dependency has been observed in the release from liposomes mediated by other cytolytic toxins such as peptide GALA (Parente *et al.*, 1990), magainin (Gomes *et al.*, 1993) and tetanus toxin (Menestrina *et al.*, 1989). For these toxins, it is postulated that low pH induces a conformation transition of the protein, exposing hydrophobic domains and making the structure better able to interact with a lipid membrane (Menestrina *et al.*, 1991). By analogy we propose that a similar process occurs for cytA27. In another study, involving the interaction of Bt cryIC protein with lipid membranes, it was found that the membrane permeabilizing activity of cryIC increases rapidly when the pH of the medium is below 5, which again, is

associated with an increased surface exposure of hydrophobic sites (Butko *et al.*, 1994). These findings are difficult to reconcile with the fact that most insect guts have a high pH (10-12). The physiological significance of the low pH induced enhancement of interaction of cytolytic proteins with lipid membranes remains enigmatic.

Further insight into the interaction of cytA proteins with lipid membranes is provided by the tryptophan fluorescence quenching experiments. Addition of lipid vesicles decreased the degree of tryptophan fluorescence quenching using either KI or acrylamide as quenchers as evidenced by the decrease in K_{eff} . This indicates that lipids bind to the proteins thereby protecting tryptophan residues from quenchers. In the absence of lipid, acrylamide accessible tryptophan residues of the cytA proteins appear to be quenched as one single fraction, $f_1 = 1$. However, in the case of the unbound cytA, for KI mediated quenching, there are two tryptophan populations, about 75% of the observed fluorescence is accessible to iodide quenching, whereas 25% is only partially accessible to iodide quenching. Upon lipid binding, about 50% of tryptophan fluorescence is accessible to iodide quenching and another 50% tryptophan fluorescence is partially accessible. The cytA24 and cytA27 proteins have an identical degree of quenching in the presence and absence of lipid, which indicates that the

tryptophan residue in the N-terminal of the cytA27 protein does not affect the fluorescence quenching property.

It seems that the mode of action of cytA proteins is quite different from Bt *cry* proteins. The currently held view for the latter is that receptors are involved in the toxin binding whereas we, and others, show that the cytA proteins can permeabilize membrane in the absence of receptors. This difference may explain the mosquitocidal toxicity of Bt subsp. *israelensis* and of other mosquitocidal Bt subspecies, e.g. Bt subsp. *darmstadiensis*, *fukuokaensis*, etc. since the crystals of these Bt subspecies all contain cytA-like proteins. Our results do not explain why the cytA proteins are toxic to a wide range of eukaryotic cells and induce leakage of artificial phospholipid vesicles *in vitro* yet *in vivo* are relatively benign to most organisms, with just specific toxicity to dipteran larvae.

In order to elucidate further the mode of action of the cytA proteins, future studies are required to investigate whether cytA proteins actually penetrate into lipid membranes and undergo conformational change upon binding to lipids.

References:

- Adang, M.J., Staver, M.J., Rocheleau, T.A., Leighton, J., Baker, R.F. and Thompson, D.V. 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. *Gene*, 36:289-300.
- Adang, M.J., Firoozabady, E., Klein, J., DeBoer, D., Sekar, V., Kemp, J.D., Murray, E., Rocheleau, T.A., Rashka, K., Staffeld, G., Stock, C., Sutton, D. and Merlo, D.J., 1987. Expression of a *Bacillus thuringiensis* insecticidal crystal protein gene in tobacco plants. In 'Molecular Strategies for Crop Protection', pp.345-353. (Alan R. Liss.)
- Adang, M.J., 1991. In 'Biotechnology for biological control of pests and vectors.' p3. (Ed. Maramorosch, K.) CRC press, Boca Raton, FL.
- Allen, T.M., 1984. Calcein as a tool in liposome methodology. In 'Liposome Technology' Vol.III, pp. 177-182, (Ed. Gregoriades, G.) CRC Press, Boca Raton, FL.
- Ames, B.N., 1966. Assays of inorganic phosphate, total phosphate and phosphatases. In 'Methods in Enzymology.' Volume VIII: Complex carbohydrates. pp. 115-118. (Eds. Neufeld, E.F. and Ginsburg, V.) Academic press, New York
- Angus, T.A., 1954. A bacterial toxin paralyzing silkworm larvae. *Nature*, 173:545-546.
- Angus, T.A., 1956. Extraction, purification and properties of *Bacillus sotto* toxin. *Can. J. Microbiol.*, 2:416-426.
- Armstrong, J.L., Rohrmann, G.F. and Beaudreau, G.S., 1985. Delta endotoxin of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.*, 161:39-46.
- Bartlet, R.J., McGuire, M.R. and Black, D.A., 1990. Feeding stimulants for the European Corn Borer (*Lepidoptera Pyralidae*): additives to a starch-based formulation of *Bacillus thuringiensis*. *Environ. Ent.*, 19:182-189.
- Barton, K.A., Whiteley, H.R. and Yang, N., 1987. *Bacillus thuringiensis* delta-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. *Plant Physiol.*, 85:1103-1109.
- Bassand, D., Jellis, C.L. and Piot, J.C., 1989. Application des techniques d'échange et de génie génétiques a l'amélioration des propriétés insecticides de *Bacillus thuringiensis*. *C. R. Acad. Agric. Fr.*, 75:127-143.
- Beegle, C.C., Couch, T.L., Alls, R.T., Versoi, P.L. and Lee, B.L., 1986. Standardization of

- HD-1-S-1980: US standard for assay of lepidopterons-active *Bacillus thuringiensis*. Bull. Entomol. Soc. Am., 32:44-45.
- Beegle, C.C., and Yamamoto, T., 1992. Invitation paper (C.P. Alexander Fund): History of *Bacillus thuringiensis* Berliner Research and Development. Can. Ent., 124:587-616.
- Berliner, E., 1911. Über die Schalfsucht der Mehlmottenraupe. Z. *Gesamte Getreidewesen* (Berlin), 3:63-70.
- Berliner, E., 1915. Über die Schalfsucht der Mehlmottenraupe. Z. *angew. Ent.*, 2:29-56.
- Bossé, M., Masson, L., and Brousseau, R., 1990. Nucleotide sequence of a novel crystal protein gene isolated from *Bacillus thuringiensis* subspecies *Kenyae*. Nucl. Acids Res., 18:7443.
- Bourgouin, C., Klier, A. and Rapoport, G., 1986. Characterization of the genes encoding the haemolytic toxin and the mosquitocidal delta-endotoxin of *Bacillus thuringiensis israelensis*. Mol. Gen. Gent., 205:390-397.
- Briggs, J.D., 1986. Pioneering and advanced phases of commercial use of *Bacillus thuringiensis* in North America. In 'Mitteilungen aus der Biologischen Bundesanstalt für land-und Forstwirtschaft Berlin-Dahlem' Vol. 233, pp25-35. (Eds. A. Krieg and A.M. Huger) Paul Parcey, Berlin.
- Burges, H.D., 1966. Mimeo, report of the Second International Symposium on the Standardization of *Bacillus thuringiensis*: Suggested Resolutions, Tests, and Principles. Wageningen, Netherlands. pp.2
- Burges, H.D., 1967. The standardization of products based on *Bacillus thuringiensis*. In 'Insect pathology and microbial control' pp. 306-314, (Ed. P. A. van der laan) North Holland Publ. Co., Amsterdam.
- Burges, H.D. and Daoust, R.A., 1986. Current status of the use of bacteria as biocontrol agents. In 'Fundamental and applied aspects of Invertebrate pathology' pp.514-517 (Eds. Samson, R.A., Vlak, J.M. and Peters, D.)
- Butko, P., Cournoyer, M., Pusztai-Carey, M. and Surewicz, W.K., 1994. Membrane interaction and surface hydrophobicity of *Bacillus thuringiensis* δ -endotoxin CryIC. FEBS letters, 340:89-92.
- Cantor, C.R. and Schimmel, P.R., 1980. Estimates of protein concentration from UV absorbance. In 'Biophysical Chemistry. Part II: Techniques for the study of biological structure and function' pp.380. W. H. Freeman and Company, San Francisco, California.

- Carlton, B.C., 1988. Genetic improvement of *Bacillus thuringiensis* as a bioinsecticide. In 'Biotechnology, Biological Pesticides and Novel Plant Pest Resistant for Insect Pest Management' pp. 38-43. (Boyce Thompson Institute, Ithaca, NY)
- Chambers, J.A., Jelen, A., Gilbert, M.P., Jany, C.S., Johnson, T.B. and Gawron-Burke, C., 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subs. *aizawai*. J. Bacteriol., 173:3966-3976.
- Chestukhina, G.G., Zalunin, I.A. Kostina, L. I., Bormatova, M.E., Klepikova, F.S., Khodova, O.M. and Stepanov, V.M. 1985. Structural features of crystal-forming proteins produced by *Bacillus thuringiensis* subspecies *israelensis*. FEBS lett., 190:345-348.
- Cheung, P.Y.K, Roe, R.M., Hammock, B.D., Judson, C.L. and Montague, M.A., 1985. The apparent *in vivo* neuromuscular effects of the δ -endotoxin of *Bacillus thuringiensis* var. *israelensis* in mice and insects of four orders. Pest Biochem. Physiol., 23:85-94.
- Chilcott, C.N., Kalmakoff, J. and Pillai, J.S., 1983. Characterization of proteolytic activity associated with *Bacillus thuringiensis* var. *israelensis* crystals. FEMS Microbiology Letters, 18:37-41.
- Chilcott, C.N., Kalmakoff, J. and Pillai, J.S., 1984. Neurotoxic and haemolytic activity of a protein isolated from *Bacillus thuringiensis* var. *israelensis* crystals. FEMS Microbiology Letters, 25:259-263.
- Chilcott, C.N. and Ellar, D.J., 1988. Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. Journal of General Microbiology, 134:2551-2558.
- Choma, C. T., Surewicz, W. K., Carey, P. R., Pozsgay, M., Raynor, T. and Kaplan, H., 1990. Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis*, structural implications. Eur. J. Biochem., 189:523-527.
- Choma, C.T., Surewicz, W. K. and Kaplan, H., 1991. The toxic moiety of the *Bacillus thuringiensis* protoxin, undergoes a conformational change upon activation. Biochem. Biophys. Res. Commun., 179: 933-938.
- Convents, D., Houssier, C., Lasters, I. and Lauwereys, M., 1990. The *Bacillus thuringiensis* delta-endotoxin: evidence for a two domain structure of the minimal toxic fragment. J. Biol. Chem., 265:1369-1375.
- Convents, D., Cherlet, M., van Damme, J., Laster, I. and Lauwereys, M., 1991. Two structural domains as a general fold of the toxic fragment of the *Bacillus thuringiensis* delta-endotoxins. Eur. J. Biochem., 195:631-635.

- Davidson, E.W., and Yamamoto, T., 1984. Isolation and assay of the toxic component from the crystals of *Bacillus thuringiensis* var. *israelensis*. *Curr. Microbiol.*, 11:171-174.
- de Barjac, H. and Lemille, F., 1970. Presence of flagellar antigenic subfactors in Serotype 3 of *Bacillus thuringiensis*. *J. Invertebr. Pathol.*, 15: 139-140.
- DeGrado, W.F., Musso, G.F., Lieber, M., Kaiser, E.T. and Kézdy, F.J., 1982. Kinetics and mechanism of haemolysis induced by melittin and by a synthetic melittin analog. *Biophysical J.*, 37:329-338.
- Delécluse, A., Charles, J., Klier, A. and Rapoport, G., 1991. Deletion by *in vivo* recombination shows that the 28-kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. *J. Bacteriol.*, 173:3374-3381.
- Dow, J.A.T., 1984. Extremely high pH in biological systems: a model for carbonate transport. *Am. J. Physiol.*, 246:R633-636.
- Dow, J.A.T., 1986. Insect midgut function. *Adv. Insect Physiol.*, 19:187-328.
- Drobniewski, F.A., Sawyer, T.J. and Ellar, D.J., 1986. The mode of action of the mosquitocidal δ -endotoxin of *Bacillus thuringiensis* var. *darmstadiensis*. In 'Bacterial protein toxins' suppl.15, pp.55-56. (Eds. Falmagne, P., Alouf, J.E., Jeljaszewicz, F.J. and Thelestam, M.) Gustav Fisher Verlag, Stuttgart.
- Drobniewski, F.A. Knowles, B.H. and Ellar, D.J., 1987. Nonspecific ionic effects on the cytolytic and haemolytic properties of *Bacillus thuringiensis* δ -endotoxins. *Curr. Microbiol.*, 15:295-299.
- Drobniewski, F.A. and Ellar, D.J., 1988a. Toxin-membrane interactions of *Bacillus thuringiensis* δ -endotoxin. *Biochemical society transactions.*, 16:39-40.
- Drobniewski, F.A. and Ellar, D.J., 1988b. Investigation of the membrane lesion induced *in vitro* by two mosquitocidal δ -endotoxins of *Bacillus thuringiensis*. *Curr. Microbiol.*, 16:195-199.
- Drobniewski, F.A. and Ellar, D.J. 1989. Purification and properties of a 28-kilodalton haemolytic and mosquitocidal protein toxin of *Bacillus thuringiensis* subsp. *darmstandiesis* 73-E10-2. *J. Bacteriol.*, 171: 3060-3067.
- Dulmage, H.T., 1970. Insecticidal activity of HD-1 , a new isolate of *Bacillus thuringiensis* var. *alesti*. *J. Invertebr. pathol.*, 15:232-239.
- Dulmage, H.T., Boening, O.P., Rehnborg, C.S. and Hansen, G.D., 1971. A proposed

- standardized bioassay for formulations of *Bacillus thuringiensis* based on the International unit. *J. Invert. Pathol.*, 18:240-245.
- Duncan, J.L. and Buckingham, L., 1981. Effect of Streptolysin S on liposomes: Influence of membrane lipid composition on toxin action. *Biochimica et Biophysica Acta*, 648:6-12.
- English, L.H., Readdy, T.R. and Bastian, A.L., 1991. Delta-endotoxin-induced leakage of $^{86}\text{R}^+$ - K^+ and H_2O from phospholipid vesicles is catalyzed by reconstituted midgut membrane. *Insect Biochem.*, 21:177-184.
- Federici, B.A., Lüthy, P. and Ibarra, J.E., 1990. The parasporal body of *Bacillus thuringiensis israelensis*: structure, protein composition, and toxicity. In 'Bacterial Control of Mosquitoes and Blackflies: Biochemistry, Genetics, and Applications of *Bacillus thuringiensis* and *Bacillus sphaericus*' Chapter 3, pp.16-44. (Eds de Barjac, H. and Sutherland, D.) Rutgers University Press, New Brunswick, New Jersey.
- Feitelson, J.S., Payne, J., and Kim, L., 1992. *Bacillus thuringiensis*: Insects and Beyond. *Bio/technology*, 10:271-275.
- Fischhoff, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.M., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G. and Fraley, R.T., 1987. Insect tolerant transgenic tomato plants. *Bio/technology*, 5:807-813.
- Gawron-Burke, C.M., Chambers, J., Jelen, A., Donovan, W., Rupar, M., Jany, C., Slaney, A., Baum, J., English, L. and Johnson, T., 1990. Molecular biology and genetics of *Bacillus thuringiensis*. In 'Proceeding Vth International Colloquium on Invertebrate Pathology and Microbial Control' pp.456-460. Adelaide, Australia.
- Gazit, E. and Shai, Y., 1993. Structural characterization, membrane interaction, and specific assembly within phospholipid membrane of hydrophobic segments from *Bacillus thuringiensis* var. *israelensis* cytolytic toxin. *Biochemistry*, 32:12363-12371.
- Gill, S.S., Cowles, E.A. and Pietrantonio, P.V., 1992. The mode of action of *Bacillus thuringiensis* endotoxins. *Annu. Rev. Entomol.*, 37:615-636.
- Gomes, A.V., de Waal, A., Berden, J.A. and Westerhoff, H.V., 1993. Electric potentiation, cooperativity, and synergism of magainin peptides in protein-free liposomes. *Biochemistry*, 32:5365-5372.
- Gonzalez, J.M., Jr., Dulmage, H.T. and Carlton, B.C. 1981. Correlation between specific plasmids and delta-endotoxin production in *Bacillus thuringiensis*. *Plasmid*, 11: 28-38.
- Gonzalez, J. M., Brown, B.J. and Carlton, B.C., 1982. Transfer of *Bacillus thuringiensis*

- plasmids coding for delta-endotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc.Natl. Acad. Sci. USA, 79:6951-6955.
- Grant, E., Jr., Beeler, T.J., Taylor, K.M.P., Gable, K. and Roseman, M.A., 1992. Mechanism of magainin 2a induced permeabilization of phospholipid vesicles. Biochemistry, 31:9912-9918.
- Haider, M.Z. and Ellar, D.J., 1987. Analysis of the molecular basis of insecticidal specificity of *Bacillus thuringiensis* crystal delta-endotoxins. Biochem. J., 248:197-201.
- Hannay, C.L., 1953. Crystalline inclusions in aerobic sporeforming bacteria. Nature, 172:1004.
- Heierson, A., Landén, R., Lövgren, A., Dalhammer, G. and Boman, H.G., 1987. Transformation of vegetative cells of *Bacillus thuringiensis* by plasmid DNA. J. Bacteriol., 169:1147-1152.
- Held, G.A., Huang, Y.S. and Kawanishi, C.Y., 1986. Effect of removal of the cytolytic factor of *Bacillus thuringiensis* subsp. *israelensis* on mosquito toxicity. Biochem. Biophys. Res. Commun., 141:937-941.
- Hofmann, C., Vanderbruggen, H., Höfte, H., Van Rie, J., Jansens, S., and Van Mellaert, H., 1988. Specificity of *Bacillus thuringiensis* delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts, Proc. Natl. Acad. Sci. USA, 85:7844-7848.
- Höfte, H., and Whiteley, H.R., 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. FEMS Microbiol. Rev., 53:242-255.
- Honee, G., Vriezen, W. and Visser, B., 1990. A translation fusion product of two different insecticidal crystal protein genes of *Bacillus thuringiensis* exhibits an enlarged insecticidal spectrum. Appl. Environ. Microbiol., 156:823-825.
- Hurley, J.M., Lee, S.G., Andrews, R.E., Jr., Klowden, M.J. and Bulla, I.A., Jr., 1985. Separation of the cytolytic and mosquitocidal proteins of *Bacillus thuringiensis* subsp. *israelensis*. Biochem. Biophys. Res. Commun., 126:961-965.
- Insell, J.P. and Fitz-James, P.C., 1985. Composition and toxicity of the inclusion of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol., 50:56-62.
- Ishiwata, S., 1901. On a kind of severe flacherie (sotto disease) (No. 1). Dainihon Sanshi Kaiho [Rep. Sericult. Assoc. Jpn.], 114:1-5 [Japanese]
- Ishiwata, S., 1905. About 'sottokin,' a bacillus of a disease of the silk-worm. Dainihon

- Sanshi Kaiho [Rep. Sericult. Assoc. Jpn.], 160:1-8 [Japanese]
- Kleir, A., Fargette, F., Ribier, J., Hoch, J., Aronson, A.I., and Minnich, S.A., 1982. Cloning and localization of the lepidopteran protoxin gene of *Bacillus thuringiensis* subsp. *kurstaki*. Proc. Natl. Acad. Sci. USA, 79:6065-6069.
- Knowles, B.H., Thomas, W.E. and Ellar, D.J., 1984. Lectin-like binding of a *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action. FEBS Lett., 168:197-202.
- Knowles, B.H. and Ellar, D.J., 1986. Characterisation and partial purification of a plasma membrane receptor for *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific δ -endotoxin. J. Cell Sci., 83:89-101.
- Knowles, B.H. and Ellar, D.H., 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ -endotoxins with different insect specificity. Biochimica et Biophysica Acta, 924:509-518.
- Knowles, B.H., Blatt, M.R., Tester, M., Horsnell, J.M., Carroll, J., Menestrina, G. and Ellar, D.J., 1989. A cytolytic δ -endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers. FEBS Lett., 244:259-262.
- Knowles, B.H., Knight, P.J.K. and Ellar, D.J., 1991. N-acetyl galactosamine is part of the receptor in insect gut epithelia that recognises an insecticidal protein from *Bacillus thuringiensis*. Proc. Roy. Soc. Lond. B, 245:31-35.
- Knowles, B. H., White, P.J., Nicholls, C.N. and Ellar, D.J., 1992. A broad-spectrum cytolytic toxin from *Bacillus thuringiensis* var. *kyushuensis*. Proc. R. Soc. Lond. B., 248:1-7.
- Knowles, B.H. and Dow, J.A.T., 1993. The crystal δ -endotoxins of *Bacillus thuringiensis*: models for their mechanism of action on the insect gut. BioEssays, 15:469-476.
- Lakowicz, J.R., 1983. Quenching of fluorescence. In 'Principles of fluorescence spectroscopy' pp. 257 - 301 (Plenum Press, New York)
- Lee, S.G., Eckblad, W. and Bulla, L.A., Jr., 1985. Diversity of protein inclusion bodies and identification of mosquitocidal protein in *Bacillus thuringiensis* subsp. *israelensis*. Biochem. Biophys. Res. Commun., 126:953-960.
- Lereclus, D., Lecadet, M.-M., Ribier, J. and Dedonder, R., 1982. Molecular relationships among plasmids of *Bacillus thuringiensis*: conserved sequences through 11 crystalliferous strains. Mol. Gen. Genet., 186:391-398.

- Lereclus, D., Delécluse and Lecadet, M., 1993. Diversity of *Bacillus thuringiensis* Toxins and Genes. In '*Bacillus thuringiensis*, An Environmental Biopesticide: Theory and Practice' pp. 37-69. (Eds. Entwistle, P.F., Cory, J.S., Bailey, M.J. and Higgs, S.) John Wiley & Sons, England
- Li, J., Carroll, J. and Ellar, D. J., 1991. Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. *Nature (London)*, 353:815-821.
- MacIntosh, S. C., Kishore, G.M., Perlak, F.J., Marrone, P.G., Stone, T.B., Sims, S.R. and Fuchs, R.L., 1990. Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. *J. Agric. Food Chem.*, 38:1145-1152.
- Maddrell, S.H.P., Lane, N.J., Harrison, J.B., Overton, J.A. and Moreton, R.B., 1988. The initial stages in the action of an insecticidal δ -endotoxin of *Bacillus thuringiensis* var. *israelensis* on the epithelial cells of the malpighian tubules of the insect, *Rhodnius prolixus*. *Journal of cell science*, 90:131-144.
- Matsuzaki, K., Nakai, S., Handa, T., Takaishi, Y., Fujita, T. and Miyajima, K., 1989. Hypelcin A, an α -Aminoisobutyric Acid containing antibiotic peptide, induced permeability change of phosphatidylcholine bilayers. *Biochemistry*, 28:9392-9398.
- McLinden, J.H., Sabourin, J.R., Clark, B.D., Gensler, D.R., Workman, W.E., and Dean, D.H., 1985. Cloning and expression of an insecticidal k-73 type crystal protein gene from *Bacillus thuringiensis* var. *kurstaki* into *Escherichia coli*. *Appl. Env. Microbiol.*, 50:623-628.
- McPherson, A., Jumak, F., Singh, G.J.P. and Gill, S.S., 1987. Preliminary X-ray diffraction analysis of crystals of *Bacillus thuringiensis* toxin, a cell membrane disrupting protein. *J. Mo. Biol.*, 195:755-757.
- Meeusen, R.L. and Warren, G., 1989. Insect control with genetically engineered crops. *Ann. Rev. Entomol.*, 34:373-381.
- Menestrina, G., 1986. Ionic channels formed by *Staphylococcus aureus* alpha-toxin: Voltage-dependent inhibition by divalent and trivalent cations. *J. Membrane Biol.*, 90:177-190.
- Menestrina, G., Forti, S., and Gambale, F., 1989. Interaction of tetanus toxin with lipid vesicles: Effects of pH, surface charge, and transmembrane potential on the kinetics of channel formation. *Biophys. J.*, 55:393-405.
- Menestrina, G., Pederzoli, C., Forti, S. and Gambale, F., 1991. Lipid interaction of *Pseudomonas aeruginosa* exotoxin A: Acid-triggered permeabilization and aggregation of lipid vesicles. *Biophys. J.* 60:1388-1400.

- Nagawa, Y. and Regen, S.L., 1992. Surfactant-induced release from phosphatidylcholine vesicles. Regulation of rupture and leakage pathways by membrane packing. *J. Am. Chem. Soc.*, 114:1668-1672.
- Oddou, P., Hartmann, H. and Geiser, M., 1991. Identification and characterization of *Heliothis virescens* midgut membrane proteins binding *Bacillus thuringiensis* δ -endotoxins. *Eur. J. Biochem.*, 202:673-680.
- Ostolaza, H., Bartolome, B., de Zarate, I.O., de la Cruz, F. and Goni, F.M., 1993. Release of lipid vesicle contents by the bacterial protein toxin α -haemolysin. *Biochimica et Biophysica Acta*, 1147:81-88.
- Parente, R.A., Nir, S. and Szoka, F.C., Jr., 1990. Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry*, 29:8720-8728.
- Paternostre, M.T., Roux, M., Rigaud, J.L., 1988. Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. I. Solubilization of large unilamellar liposome (prepared by reverse-phase evaporation) by Triton X-100, octyl glucoside, and sodium cholate. *Biochemistry*, 27:2668-2677.
- Perlak, F.J., Deaton, R.W., Armstrong, T.A., Fuchs, R.L., Sims, S.R., Greenplate, J.T. and Fischhoff, D.A., 1990. Insect resistant cotton plants. *Bio/technology*, 8:939-943.
- Scharff, T.G. and Maupin, W.C., 1960. Correlation of the metabolic effects of benzalkonium chloride with its membrane effects in yeast. *Biochem. Pharmacol.*, 5:79-86.
- Schnepf, H.E. and Whiteley, H.R., 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, 78:2893-2897.
- Schnepf, H. E., Wong, H.C. and Whiteley, H.R., 1985. The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *J. Biol. Chem.*, 260:6264-6272.
- Sick, A., Gaertner, F. and Wong, A., 1990. Nucleotide sequence of a coleopteran-active toxin gene from a new isolate of *Bacillus thuringiensis* subs. *tolworthi*. *Nucl. Acids res.*, 18:1305.
- Silhankova, L., 1959. Effect of dimethyldodecylbenzylammonium chloride (Ajatin) on the resistance of cell membranes in rough and smooth forms of yeast cells. *Folio microbiol.*, 4:29-40.
- Slatin, S.L., Abrams, C.K. and English, L., 1990. Delta-endotoxins form cation-selective

- channels in planar lipid bilayer. *Biochem. Biophys. Res. Commun.*, 169:765-772.
- Steinhaus, E.A., 1951. Possible use of *Bacillus thuringiensis* Berliner as an aid in the biological control of the alfalfa caterpillar. *Hilgardia*, 20:359-381.
- Steinhaus, E.A., 1956. Potentialities for microbial control of insects. *J. Agric. Food Chem.*, 4:676-680.
- Taylor, R., Tippett, J., Gibb, G., Pells, S., Pike, D., Jordan, L. and Ely, S., 1992. Identification and characterization of a novel *Bacillus thuringiensis* δ -endotoxin entomocidal to coleopteran and lepidopteran larvae. *Mol. Microbiol.*, 6:1211-1217.
- Terwilliger, T.C., Weissman, L. and Eisenberg, D., 1982. The structure of melittin in the form I crystals and its implications for melittin's lytic and surface activities. *Biophys. J.*, 37:353-361.
- Thomas, W.E. and Ellar, D.J., 1983a. *Bacillus thuringiensis* var. *israelensis* crystal δ -endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. *J. Cell Sci.*, 60:181-197.
- Thomas, W.E. and Ellar, D.J., 1983b. Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal δ -endotoxin. *FEBS letters*, 154:362-368.
- Vaeck, M., Reynaerts, A., Höfte, H., Jansens, S., de Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M. and Leemans, J., 1987. Transgenic plants protected from insect attack. *Nature(London)*, 328:33-37.
- Van Frankenhuyzen, K., 1993. The challenge of *Bacillus thuringiensis*. In '*Bacillus thuringiensis*, An Environmental Biopesticide: Theory and Practice.' pp 1-35 (Eds. Entwistle, P.F., Cory, J.S., Bailey, M.J. and Higgs, S.) John Wiley & Sons (England).
- Van Rie, J., Jansens, S., Höfte, H., Degheele, D. and Van Mellaert, H., 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. *Appl. Env. Microbiol.*, 56:1378-1385.
- Visser, B., Munsterman, E., Stoker, A. and Dirkse, W.G., 1990. A novel *Bacillus thuringiensis* gene encoding a *Spodoptera exigua*-specific crystal protein. *J. Bacteriol.*, 172:6783-6788.
- Visser, B., Bosck, D., and Honée, G., 1993. Domain-function studies of *Bacillus thuringiensis* crystal proteins: A genetic approach. In '*Bacillus thuringiensis*, An Environmental Biopesticide: Theory and Practice' pp.71-88. (Eds. Entwistle, P.F., Cory, J.S., Bailey, M.J. and Higgs, S.) John Wiley & Sons, England

- Waalwijk, C., Dullemans, A.M., van Workum, M.E. and Visser, B., 1985. Molecular cloning and the nucleotide sequence of the Mt 28,000 crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. *Nucleic Acids Res.*, 13, 8207-8217.
- Ward, E.S., Ellar, D.J. and Chilcott, C.N., 1988. Single amino acid changes in the *Bacillus thuringiensis* var. *israelensis* δ -endotoxin affect the toxicity and expression of the protein. *J. Mol. Biol.*, 202:527-535.
- Weinstein, J.N., Ralston, E., Leserman, L.D., Klausner, R. D., Dragsten, P., Henkart, P., and Blumenthal, R., 1984. Self-quenching of carboxyfluorescein fluorescence: uses in studying liposome stability and liposome-cell interaction. 'In Liposome Technology' (Ed. Gregoriades, G.) Vol. III, pp. 183-204. CRC Press, Boca Raton, FL.
- Weiser, J., 1986. Impact of *Bacillus thuringiensis* on applied entomology in eastern Europe and in the Soviet Union. In 'Mitteilungen aus der Biologischen Bundesanstalt für land-und Forstwirtschaft Berlin-Dahlem' (Eds. A. Krieg and A.M. Huger) Vol. 233, pp. 37-50, Paul parey, Berlin.
- Whiteley, H. R., and Schnepf, H.E., 1986. The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. *Ann. Rev. Microbiol.*, 40:549-576.
- Wu, D. and Chang, F.N., 1985. Synergism in mosquitocidal activity of 26 and 65 kDa proteins of *Bacillus thuringiensis* subsp. *israelensis* crystal. *FEBS Lett.*, 190:232-236.
- Yu, Y. M., Ohba, M. and Gill, S. S., 1991. Characterization of mosquitocidal activity of *Bacillus thuringiensis* subsp. *fukuokaensis* crystal proteins. *Appl. Environ. Microbiol.*, 57:1075-1081.
- Zakharyan, R.A., Agabalyan, A.S., Chil-Akopyan, L.A., Gasparyan, N.S., Bakunts, K.A., Tatevosyan, P.A. and Afirkyan, E.K., 1976. About the possibility of extrachromosomal DNA in creation of the entomocidal endotoxin of *B. thuringiensis*. *Dokl. Akad. Nauk. Arrn. SSR.*, 63:42-47. [In Russian].