

To the Graduate School:

I am submitting herewith a dissertation written by Stephen Robert Grant entitled "Site-Specific Binding of Lipid Vesicles." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Biochemistry.

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SITE-SPECIFIC BINDING OF LIPID VESICLES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Stephen Robert Grant

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DEDICATION

This manuscript is dedicated to my wife, Jean. This dedication is based upon the total love, hard work, emotional and financial support and the ten years of giving up of all earthly possessions. She has been the central theme of my life for over 15 years. We have taken on our lives as a partnership. I cannot express the love and admiration I feel for this person. She is certainly a heroine in my eyes. I am certain that Jean's guidance and drive are as much a part of this manuscript as are mine. I know in my heart that the accomplishments set forth from this manuscript have to be shared equally, as we have always done.

Many times over the last decade I felt the goals were too high; when I was defeated, disheartened and discouraged, Jean's drive and ambition sparked my heart and I knew I must continue. I love you, now, more than ever. Please accept this manuscript in turn for all of the hardships it has cost.

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ABSTRACT

This dissertation describes an attempt to produce a model system which will optimize the experimental conditions for site-specific lipid vesicle binding in vitro. This model system uses a modified ligand protein highly specific for binding to a distinct class of receptors and incorporates this protein into the bilayer of various liposome preparations. Two synthetic reactions were used to modify the ligand.

In the first approach, octanoic acid anhydride was synthesized and used as a coupling reagent for the incorporation of the octanoic acid moiety to the α -bungarotoxin molecules through an amide bond. The isolated reaction product, mono-octanoyl-bungarotoxin (OBGT) was synthesized in sufficient yield (15%) and in high purity. This new modified ligand was then examined for its affinity to bind to lipid vesicles ($K_D \sim 10^{-7}$ M). OBGT was then evaluated for its binding properties to the acetylcholine receptor solubilized from Torpedo californica ($K_D \sim 10^{-8}$ M). Finally, OBGT was incorporated into vesicles which were then examined with respect to their site-specific binding to the microsac membranes enriched with the acetylcholine receptors. This binding was four times higher than the protein-free vesicles demonstrating a dramatic increase in vesicle affinity for microsacs.

The second approach involved the synthesis of a modified α BGT which has one palmitic acid moiety covalently attached through an amide bond. This reaction was accomplished by using N-hydroxy

succinimide ester of palmitic acid. This coupling reagent proved superior to the fatty acid anhydride reaction used earlier due to the increased yield ($\geq 60\%$) of modified toxin. This reaction could also be better controlled and therefore a larger percentage of the α BGT was converted into mono-palmitoyl-bungarotoxin (PBGT). This product was purified to a high degree of homogeneity. Essentially all of the PBGT became vesicle associated. PBGT had a K_D of about 10^{-7} M for the detergent solubilized receptor as compared to 10^{-8} M for α BGT. The site-specific binding of vesicles containing PBGT was analyzed in detail. The results showed that PBGT-vesicles obeyed the principles of multivalent binding. There was a threshold value of about 300 PBGT molecules per vesicle, above which the site-specific vesicle binding rapidly increased.

The results of site specific vesicle binding were evaluated with respect to the principles of the multivalent binding hypothesis. The two coupling reactions were compared in terms of their contributions to progress in liposome research. The results focused on important restrictions and defined limits of density for membrane ligands of vesicles involved in many basic areas of liposome research, including the membrane-membrane interaction mediated by specific ligand-receptor binding, and the site-specific drug delivery to tumor cells.

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Cell Ghost Membranes 65

14. Binding of PBGT-Vesicles to Microsac or Red Blood

Cell Ghost Membranes over the Entire Concentration

Range 68

LIST OF ABBREVIATIONS

LUV	Large unilamellar vesicles
SUV	Small unilamellar vesicles
MLV	Multilamellar vesicles
α BGT	α -bungarotoxin
EYPC	Egg yolk phosphatidylcholine
DOPC	Dioleoyl phosphatidylcholine
EGTA	Ethylenebis-(oxyethylene nitrilo) tetraacetic acid
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
AchR	Acetylcholine receptor
OBGT	Mono-octanoyl- α -bungarotoxin
RBCG	Red blood cell ghost
SDS	Sodium dodecyl sulfate
β BGT	β -bungarotoxin
DOL	Dioleoyl lecithin
DOC	Deoxycholate
PBGT	Palmitoyl-bungarotoxin
PMSF	Phenylmethyl sulfonyl fluoride
EDTA	Disodium ethylene diamine
TSTR	Triton X-100 solubilized tetraacetate torpedo receptor
BSA	Bovine serum albumin
MOPS	3-(N-morpholino propane sulfonic acid)
NHSP	N-hydroxysuccinimide ester of palmitic acid

CHAPTER I

INTRODUCTION

Historical Perspective

This year marks the beginning of the second decade in which lipid vesicles, liposomes (1) have been used as carriers for drug therapy.* The first definitive study using lipid vesicles as drug carriers was reported by Magee and Miller in 1972 (2). In 1975, Weissman et al. demonstrated that the biological molecules carried within the liposome can be delivered directly into the cytoplasm of the cell (3). Both of these early reports show the usefulness of lipid vesicles as a delivery system.

Since those two early reports, the use of liposomes in general membrane research has experienced dramatic growth. Liposomes as drug carriers and their interaction with cells has been the subject of several reviews (4-11) and at least one text (12). Recently, a review on the subject of methods of preparation of liposomes and their comparative properties gave an excellent description into the classification of individual liposomes (13). Multilamellar vesicles (MLV) were used extensively in this earlier work. However, because of its

*When referring to lipid vesicles the nomenclature that was informally agreed upon at the New York Academy of Sciences meeting on "Liposomes and Their Uses in Biology and Medicine" is used. In this nomenclature, all types of lipid bilayers surrounding an aqueous space are considered to be in the general category of liposomes. The different types of liposomes are then referred to by a three letter acronym. The classes are (i) multilamellar vesicles (MLV), (ii) small (<1000Å) unilamellar vesicles (SUV) and (iii) large (>1000Å) unilamellar vesicles (LUV).

alternating layers of lipid and aqueous volume, it was difficult to assess the type of interaction between it and cells. Therefore, more recent research has made use of the unilamellar type of vesicle. These unilamellar structures, (i) small unilamellar vesicles, SUV, and (ii) large unilamellar vesicles, LUV, offered several distinct advantages over MLV.

1. They were relatively uniform in size (if proper care was exercised during their formation).
2. The unilamellar construction permitted direct evaluation of the type of interaction between the vesicle and the cell (fusion, endocytosis, or absorption).
3. It was much easier to evaluate the fate of both the bilayer and the trapped content radiolabeled marker during vesicle-cell interaction.

There were two basic types of delivery systems for drug therapy. One type which included liposomes was the type that contained a biologically active compound by trapping it inside its structure. Porous beads, artificial cells and some polymer structures also fell into this category. The second type depended upon a direct coupling or strong affinity between the carriers and the therapeutic agent. Direct linkage of this type has been attempted for antitumor drugs to polymers, proteins, DNA, hormones and antibodies. There has also been an attempt to link antiviral compounds to dipeptides, proteins and polymers. The merits of these systems and all pertinent references have been evaluated in three recent reviews (14-16). In general, the direct linkage technique has met with only limited success. In several studies,

the evidence suggested that there was very little control over the fate of the endocytic:drug-carrier complex. More importantly, there was evidence that these complexes may be difficult to break down. A failure in the breakdown process would in most cases render the drug inactive. An exhaustive list of arguments could be presented against direct coupling depending upon both the type of carrier used and the drug to which it is attached. These problems were routinely cited in a majority of the reports exploring this approach.

In light of the problems surrounding direct linkage discussed above, liposomes offered some clear advantages. In general, liposome formation was relatively simple. It required no sophisticated equipment and lipids were generally inexpensive biochemicals. Due to their unique structure (i.e. an aqueous trapped content surrounded by a relatively impermeable lipid bilayer), they were able to contain a wide variety of biologically active compounds in relatively high concentration (17-18). There was a natural affinity of a liposome for the cell membrane. Cultured mammalian cells were known to interact readily with these structures (19-23). Entrapment required no alteration of the therapeutic agent. This was a big advantage because once the vesicle had interacted with the cell, the biologically active compound was delivered into the cell in an active unaltered state. Liposomes were capable of trapping a combination of drugs in a one-step process. Therefore, a large battery of biologically active compounds may be packaged into liposomes. Finally, SUV's and LUV's, because of their unilamellar construction, trapped these agents within a single bilayer of lipid. In the event of fusion, these drugs were immediately available for their pharmacological activity.

Although liposomes offered many advantages over the direct linkage systems, they did have problems related to drug delivery. Liposomes have a finite life span. They degrade with time releasing their trapped content or fusing their lipid bilayers. They have been made "leaky" such that the vesicle was no longer retentive to trapped content. Under these conditions, the vesicle experienced a rapid release of trapped content. Liposomes under certain conditions were susceptible to serum attack, e.g., phospholipase activity and the action of phospholipid exchange proteins. In some in vivo drug delivery experiments, problems of lipid absorption by blood components have been encountered. Several reports listed in the reviews have cited the important problems associated with the clearing of vesicles by the reticuloendothelial cells in the liver and spleen. These problems manifest themselves as very difficult barriers; however, the major problem of using liposomes in drug delivery was specificity. As already pointed out, most cells have an affinity for lipid vesicles. Therefore, liposomes would be an excellent vehicle for delivery of biologically active compounds to all cells in general. However, they were a poor means of delivery to certain cell types due to this lack of specificity. Except in the case when the cell type has an affinity for plain lipid vesicles, unmodified liposomes exhibit no structural features which would provide them with a specificity to a distinct class of cells. If liposomes were to be used as a vehicle for site-specific drug delivery, then some modifications of the lipid bilayer must be made to gain this necessary specificity.

Concept of Site-specific Drug Delivery (Targeting)

The idea of using a site-specific liposome for drug delivery has been a popular concept within the field of vesicle-cell interaction. However, to date, there are very few examples of this idea. Specificity for cell type by modified liposomes has been attempted by using specific glycoproteins (24-25), heat-aggregated antibody (26-28) and trinitro-phenyl hapten (29). In the cases of heat-aggregated antibody and antibody not covalently attached to the liposomes, enhancement of target specific delivery was only minimal (30). In other cases reported, the systems have a limited specificity to broad classes of cells or use totally artificial haptens. Nevertheless, these reports suggest that site-specific targeting of lipid vesicles was possible. I believe site-specific targeting to be the most important contribution to the field of liposome research since the first demonstration of using liposomes as a drug delivery system. I am also convinced that the best system for site-specific targeting has not been successfully attempted. This model system has been envisioned as using a modified ligand incorporated into the lipid bilayer of a vesicle for the purpose of targeting this vesicle to a specific cell receptor which was exposed on the surface membrane of some specific cell type. In using such a system, the researcher took advantage of the following characteristics of the ligand-receptor pair:

1. Each ligand-receptor pair has a built-in affinity usually several orders of magnitude greater than simple ionic or hydrophobic interactions.

2. The ligand-receptor interaction was highly specific. This interaction has been identified with a single cell type, or at worst, a single class of cells.
3. Receptor densities on the outer surface of the plasma membrane were very high in some cell types.
4. New receptors were constantly being synthesized within the cell, replacing old or damaged receptors on the cell surface.
5. Ligand densities on the vesicle surface could be varied over a wide range.
6. Many ligand molecules were relatively stable, as they were often resistant to chemical and thermal denaturation.

One excellent example of a ligand-receptor pair showing all of the above characteristics was the α -bungarotoxin-acetylcholine receptor pair. The acetylcholine receptor was the most extensively studied and best understood receptor system known in biology. Several reviews detail the vast wealth of information presently available for this receptor complex (31-34). The acetylcholine receptor has an unusually high affinity ($K_D = 10^{-11}$ M) for α BGT (35). Its surface density on the plasma membrane in some tissues was large enough to account for a majority of the membrane protein (36). This extremely high surface density is characteristic of plasma membranes from the electric organ of Torpedo californica (37). A partially purified functional acetylcholine receptor membrane vesicle preparation has been obtained with a high yield (38). These vesicles have been called microsacs because they were intact functional small ($500-2500\text{\AA}$) sack-like structures

containing a high density (1.5×10^5 receptor sites/mg of membrane protein) of acetylcholine receptor complexes (39). Microsacs were ideal structures for a model membrane system to which liposomes could be targeted via a modified α BGT molecule. The advantages of using microsacs, as opposed to other membrane preparations like whole cells, were many. Firstly, they have been isolated in high yield and good purity. Secondly, they have been stored for long periods of time without degradation. Thirdly, under specified conditions, microsacs were made a uniform size and density. This made them easy to band on sucrose gradients. Finally, microsacs were labeled with radioactive iodine using either lactoperoxidase or chloramine-T techniques. Therefore, a wide range of specific activities was obtained for the microsome preparations. All of these advantages made microsacs an ideal model of a cell surface environment.

α BGT also had distinct advantages as a model ligand. It was well known that α BGT, like all α -toxins, was a very stable macromolecule (40). Its small size (8000 daltons) and its large number of intrachain disulfide bridges (five), made the molecule very resistant to both chemical and thermal degradation (41-42). The toxin has been purified to homogeneity without great difficulty. There were several antagonists which competed against α BGT for binding to the acetylcholine receptor, thus providing well-defined pharmacological characteristics for simple identification. α BGT had one big disadvantage; it was very expensive.

Concept of Multivalent Binding, Membrane-membrane Interaction
and a Model System for Investigation

The author felt the necessity to provide a simple theoretical model based upon the biochemical and biophysical parameters of membrane-membrane interactions. This theoretical model must explain the results of such interactions in our site-specific lipid vesicle binding model. The existence of such a theoretical model had been developed several years ago to explain the interaction of an antibody molecule (bivalent) with monovalent and multivalent ligands (43-44). As the hypothesis developed, Karush defined the term "functional affinity" to describe in a quantitative manner a factor which represented the multiple by which the intrinsic affinity of the antibody molecule for its antigenic ligand was increased by virtue of multivalent interactions. From an operational point of view, this enhancement factor was evaluated from the ratio of the affinities for the interaction of a particular antibody population with a monovalent and a multivalent ligand. In particular, Fred Karush demonstrated that an anti-lactoside antibody population had an association constant order of $2 \times 10^9 \text{ M}^{-1}$ (ϕ X 174 had been conjugated with multiple lactoside groups against which the antibody was directed). When this association constant for neutralization was compared with equilibrium binding measurements of this antibody to a monovalent soluble ligand, N-acetyl-p-aminophenyl- β -lactoside, the intrinsic association constant, K_0 , was in the range of $2 \times 10^5 \text{ M}^{-1}$. A difference of the order of 10^4 existed between these two binding studies with the same antibody. The

only difference was that one system employed a multivalent ligand substrate, the lactoside group conjugated ϕ X 174 compared to the other system which employed the monovalent ligand. Therefore, this enhancement in binding (which is a ratio of these two binding constants) was attributed to the bivalent interaction of the antibody and a reasonable generalization for the numerical value of this enhancement was in the order of magnitude of 10^4 .

A natural extension of this hypothesis was provided by George Bell to explain the theoretical framework for adhesion mediated by reversible bonds between cell surface molecules. In this latest revision, multivalent binding has been extended to include the principals fundamental to any two membrane systems which interact through a ligand-receptor or receptor-receptor interaction. It became apparent that from a strictly theoretical point of view, the affinity between a targeting liposome with a cell fulfilled the conditions for multivalent binding, if multiple specific bonds were established between the liposome and the cell membrane. In an ideal case, one would like to put as many targeting molecules as possible to the surface of the liposome, thus enhancing the chance of multiple binding. This situation was a two-dimensional analogy to the base pairing of two complementary single stranded DNA's in which a strong positive cooperativity of binding exists. On a statistical basis, one could envision the establishment of the first pair of specific bonds between a targeting molecule and a binding site on the cell upon a random collision of liposome with cell should enhance the probability of the establishment of the neighboring pairs. From the dissociation

point of view, once multiple bonds are established between the liposome and the cell, the only chance of dissociation will be the simultaneous breaking of all bonds at the same time. The probability of such a situation rapidly diminished and approached zero when the number of specific bonds between the liposome and the cell increased. Thus, one predicts a positive cooperativity exists when the extent of liposome binding to the target cells is examined as a function of the density of the targeting molecule on the liposomes (45-46). A threshold density should exist, above which the multivalent binding occurs. This prediction was one of the important aspects examined and evaluated in this work.

CHAPTER II

STUDIES WITH MONO-OCTANOYL- α -BUNGAROTOXIN

Abstract

Purified α -bungarotoxin was reacted with octanoic acid anhydride in a mixed solvent system of dioxane and water. The reaction products were separated by Sephadex LH-20 and Phenyl-sepharose column chromatography. Using radioactive tracers, it was shown that mono-octanoyl- α -bungarotoxin was the major coupling product of the reaction. The purified mono-octanoyl- α -bungarotoxin bound with either sonicated or unsonicated phosphatidylcholine vesicles had a dissociation constant, K_D , of 10^{-7} M, whereas the underivatized α -bungarotoxin did not show any binding to lipid vesicles. Furthermore, the specific binding of ^{125}I - α -bungarotoxin to the detergent solubilized acetylcholine receptor from Torpedo californica was effectively inhibited by prior preincubation of mono-octanoyl- α -bungarotoxin with the receptor; >60% inhibition was observed at a 1:1 mole ratio of mono-octanoyl- α -bungarotoxin to ^{125}I - α -bungarotoxin. This increased inhibition above 50% may be the result of the hydrophobic nature of the molecule. The estimated affinity of mono-octanoyl- α -bungarotoxin to the acetylcholine receptor was about 10-fold less than the native toxin and very close to the estimated affinity of the iodinated species; however, it was still about five orders of magnitude higher than the α -toxin of the Naja naja siamensis venom. These results indicate that fatty acids could be covalently coupled to α -bungarotoxin and the derivatized toxin became an amphipathic molecule which retained considerable amounts of

the specific binding activity to the acetylcholine receptors. The use of this system as a model to study the specific interactions between membranes was discussed.

Introduction

Phospholipid vesicles (liposomes) have been used both to modify the plasma membrane composition and cell behavior, and as carriers for drugs, hormones, enzymes or other biologically active substances in vitro or in vivo (for recent reviews, see Pagano and Weinstein, 1978) (47). One of the central problems of this area of research was the lack of specificity in terms of the type of cells with which the phospholipid vesicles can interact. Attempts have been made to modify the vesicle surface membranes with some targeting molecules so that the vesicles acquired the desired specificity, a process termed liposome targeting. Immunoglobulins (48-49) and the desialated fetuin (50) have been "coated" onto the vesicle surface. The uptake of the vesicle's entrapped contents by the target cells increased significantly when compared to the non-targeted vesicles. In these studies, however, the targeting molecules were non-covalently attached to the vesicle surface. The stability of the targeting molecule was not evaluated under the in vitro or in vivo conditions when the vesicles were subsequently exposed to cells. The targeting molecules may conceivably be detached or modified under these conditions and hence reduce the efficiency of targeting. It therefore appeared attractive to explore the possibility of covalent coupling of the targeting molecule with the lipid vesicles. For this purpose, we have used the α -bungarotoxin as a model system for the targeting molecule and

covalently coupled the fatty acyl moiety to the protein. The resulting amphipathic toxin molecule was characterized in some detail and tested for liposome targeting in a model system.

Materials

Lyophilized snake venoms were purchased from Miami Serpentarium, Florida. Egg yolk phosphatidylcholine (EYPC), dioleoyl phosphatidylcholine (DOPC) and ^3H -dipalmitoyl phosphatidylcholine (^3H -DOPC) were prepared as described (51-53). All lipids were stored in sealed ampules at -20°C under N_2 . Purity was checked by thin-layer chromatography (54). ^{14}C -labeled and unlabeled octanoic acid anhydride were synthesized using dicyclohexylcarbodiimide (Eastman Kodak, Rochester, New York) and stored in anhydrous dioxane in sealed ampules (55).

Methods

Preparation of Snake Venom Toxins

The purification of α -bungarotoxin (αBGT) from lyophilized venom of Bungarus Multicinctus was accomplished using a modification of the procedure first described by C.Y. Lee et al. (56). The modifications to the procedure were as follows:

A 10 ml solution of 0.05 M ammonium acetate buffer pH 5.0 containing 0.5 gms of crude lyophilized venom was applied onto a 2.5 cm x 53 cm Sephadex CM-50 column which has been pre-equilibrated in 0.05 M ammonium acetate buffer. Elution was carried out using a 500 ml linear gradient made of equal volumes of 0.05 M ammonium acetate, pH 5.0, and 1.0 M ammonium acetate, pH 7.0, collecting 80 drops/fraction (which was equivalent to 5.0 ml fraction at 4°C). Pooled fractions representing

peak II₂ as identified by C.Y. Lee et al. (57) were concentrated to 3.0 ml under N₂ at 4°C in a 10 ml Aminco concentrator. The sample was diluted with distilled water to 20 ml and concentrated to 3 ml again. The sample volume was adjusted such that the solution yields a conductivity of less than 4.0 mmhos. This solution was then layered onto a pre-washed, pre-equilibrated Whatman CM-32 column (1.0 cm x 53 cm). The sample was then washed with 20 ml of 0.05 M ammonium acetate, pH 5.0. Elution was carried out using a 300 ml linear gradient, made of equal volumes of 0.05 M ammonium acetate, pH 5.0, and 0.3 M ammonium acetate, pH 7.0. The elution profile contains three protein peaks. The first small peak proved to be a minor contaminate. The two major peaks identified as II₁ and II₂ by C.Y. Lee were assayed for phospholipase A activity throughout the elution profile as described by K. Kondo et al., using thin layer chromatography (58). A slight modification of this technique was followed. Instead of determining the percent of liberated fatty acid by charring with 50% (w/v) H₂SO₄/H₂O and then scanning with a densitometer, the assay utilized ³H-labeled dioleoyl phosphatidylcholine and the plates were scraped and counted by liquid scineillation counting. The fractions enriched in acetylcholine receptor binding and absent of all or most phospholipase A activity were pooled and labeled II₂. These fractions were then dialyzed three times in Spectropor 3 (molecular weight cut-off 3000-5000 daltons) against PBS buffer pH 7.4 containing 5 mM EGTA. This dialysis was necessary because ammonium acetate interferes with coupling reaction due to the presence of free NH₄⁺ group. Secondly, EGTA which binds calcium specifically at low concentrations removed any available

Ca^{++} from solution. It has been shown previously that phospholipase A_2 activity was absolutely dependent upon free calcium for activity. This concentration of EGTA was 100% effective in reducing residual phospholipase A activity down to background levels. The purification of α -naja toxin from the venom of Naja naja siamensis was similarly done as for α BGT.

Preparation of ^{125}I -BGT

Iodination of α BGT was carried out by the chloramine-T method of Hunter and Greenwood (59). A typical iodination, outlined below, had slight modifications employed whenever a higher or a lower specific activity was needed. Ten mg of purified α BGT (8.0 mg/ml) in PBS-EGTA buffer pH 7.4 was placed in a 12 x 75 mm glass tube. Added to this solution were 150 μl of 2.0 M Tris buffer pH 8.2, 25 μl of KI (8.2 mg/ml), 50 μl Na ^{125}I in 0.1 N NaOH (5 mCi carrier free) and 0.5 ml PBS pH 7.4. This solution was mixed well and to it was added 0.1 ml of freshly prepared chloramine-T (2.0 mg/ml). The reaction mixture was incubated at room temperature for 45 minutes. The reaction was stopped by the addition of 100 μl $\text{Na}_2\text{S}_2\text{O}_5$ (4.0 mg/ml), followed 10 minutes later by 200 μl of KI solution (200 mg/ml). The final reaction solution was applied onto a BioGel P-4 column which had been pre-equilibrated in 0.05 M ammonium acetate pH 5.0. Fractions (0.3 ml) were collected and the void volume peak of radioactivity was pooled and layered onto a 5 ml column of Sephadex CM-50 equilibrated in 0.05 M ammonium acetate pH 5.0. This column was then washed with 10 ml of the equilibration buffer and then eluted with 10 ml of 0.5 M ammonium acetate pH 6.0. The radioactive protein peak was then dialyzed

in Spectropor 3. The dialysed solution's protein was measured by the procedure of Lowry et al. (60), using bovine serum albumin as the protein standard. The specific activity was determined by gamma counting. The protein was diluted to a convenient concentration in PBS-EGTA pH 7.4 and the specific activity of ^{125}I -BGT was typically 1.0×10^{13} cpm/mole.

Coupling of Fatty Acid to α -BGT

The method for the coupling of octanoic anhydride to toxin was a modification of the acetic acid anhydride coupling to protein. Octanoic acid anhydride (3 μl of 0.119 M in dioxane) was added to 100 μl ^{125}I - α BGT (5 mg/ml in 0.3 M sodium phosphate, pH 7.2) and the mixture was rapidly agitated in a vortex mixer for five minutes, then left standing for an additional 45 minutes at room temperature. The cloudy reaction products were dried in vacuum and resuspended in H_2O before loading to a Sephadex LH-20 column (Pharmacia, Uppsala) which was pre-equilibrated with PBS. The column was eluted with PBS and the ^{125}I radioactivity of fractions was measured in a gamma counter. The major radioactive peak containing >90% of the original radioactivity was pooled and applied to a Phenyl-sepharose column (Pharmacia, Uppsala) pre-equilibrated with PBS. Elution with PBS was continued until no ^{125}I radioactivity was further eluted. At this time, the elution solvent was changed to 70% ETOH and the elution continued until no further radioactivity appeared in the eluent. The radioactive protein, which was identified as the coupling product eluted by 70% ETOH was pooled, dried in vacuum and resuspended in PBS.

Phospholipid Vesicles

The method of Bangham et al. (61) was followed to prepare the unsonicated lipid vesicles. The procedure to prepare sonicated vesicles has been described in detail (62). In all cases, a trace of ^3H -dipalmitoyl PC was added to either EYPC or DOPC to give a final specific activity of 2.0×10^{11} cpm/mole. PBS was used as the aqueous buffer system.

Acetylcholine Receptor (AChR) Preparation

The method of Karlin and Cowburn (63) was followed to prepare the triton X-100 solubilized AChR from frozen electroplaxes of Torpedo californica (Pacific Biomarine, Venice, California). The specific activity of the solubilized AChR was 0.5 nmoles αBGT binding sites/mg total protein as assayed by a gel filtration method.

Assay of αBGT Binding to Solubilized AChR

Method 1: AChR (5 μg protein) and ^{125}I - αBGT (125 pmoles) in Tris-triton buffer (1% triton X-100 in 10 mM Tris pH 7.8) were incubated at 37°C for one hour. The mixture was then applied to a 10 ml Biogel p-60 (BioRad) column pre-equilibrated and eluted with Tris-triton buffer. The radioactivity of the toxin-receptor complex eluted at the void volume was used to calculate the total toxin binding sites of AChR.

Method 2: For routine assays of toxin-receptor binding, a modification of a filter assay first described by Devorotes and Fambrough in 1975 was used (64). We used three DE81 filters stacked under one CM32 filter. The filters were washed with 0.4 M NaPO_4 buffer, pH 5.4 containing 2.0% Triton X-100. The filters were rinsed with 20 ml of this buffer solution three times for 30 minutes,

exchanging the rinse buffer each time. The total incubation volume for the assay was 300 μ l. In these assays, 6.0 μ g solubilized AchR was incubated with 100 pmoles of ^{125} I- α BGT with or without the competing ligand at 37 C for one hour. The mixture was then assayed for ^{125} I- α BGT-AchR complex by the filter assay method.

Isolation and Radioiodination of Microsacs and Red Blood Cell Ghosts (RBCG)

The method of Miller et al. (65) was followed with minor modifications to isolate microsacs from frozen electroplaxes of Torpedo californica. The purified microsacs had a specific activity of 0.5 nmoles of total α BGT binding sites per mg protein. Ghosts of human red blood cells were prepared (66). Membrane iodination with ^{131} I was done by the lactoperoxidase-glucose oxidase method (67). The ^{131} I-membranes were washed extensively by centrifugation and dialysis to remove free ^{131} I. The final specific activity was 1.0×10^6 cpm/mg. The toxin binding activity of the microsacs was not impaired by iodination.

Binding Assay of Lipid Vesicles to Microsacs

Plain, protein-free vesicles were prepared by sonicating 20 mg ^{3} H-PC in PBS buffer pH 7.4. OBGT-vesicles were obtained in a similar manner by cosonation of 20 mg ^{3} H-PC and 0.25 OBGT. Specific activity of the lipid was 1.0×10^{12} cpm/mole in both preparations. The specific activity of the OBGT was 1.0×10^{13} cpm/mole. Unincorporated OBGT was removed as a free protein by separation on a Sephadex G-50 column. Radiolabeled microsacs and RBC ghosts (0.1 mg total protein) were incubated with vesicles (0.4 mg lipid) for 30 minutes

at 4°C. The mixture was spun in a SW50.1 rotor at 45,000 rpm ($1.9 \times 10^5 \times g$) for 135 minutes at 4°C through a 10% (w/v) sucrose in PBS solution. This procedure pelleted >90% microsac or RBC ghost protein. The pellets were washed twice in PBS before counting for ^3H , ^{125}I and ^{131}I . The extent of vesicle binding (^3H and/or ^{125}I cpm) was corrected for microsac and RBCG recovery as measured by ^{131}I -cpm.

Other Analytical Methods

Lipid phosphorus was determined by the method of Ames and Dubin (69). ^{125}I and ^{131}I were counted in a Beckman Biogamma II counter. ^{14}C and ^3H were counted in a Beckman LS-230 liquid scintillation counter using a triton X-100 containing cocktail. All results were corrected for channel crossover if more than one isotope was used.

RESULTS

Purification of α BGT from Crude Venom of Bungarus Multicinctus

The elution profile and the purification of α -bungarotoxin can be seen in Figure 1. The pooled fractions from the CM-50 column were concentrated and the ionic strength diluted as described in Methods (page 13). This solution was then placed on a CM-32 Whatman column and eluted. The first protein peak was a minor contaminate and was discarded. The remaining two major protein peaks corresponded to the labeled peaks II_1 and II_2 described by C.Y. Lee (see Methods). Pooled fractions 108-118 (peak II_2) showed one band (8000 dalton) in 12% SDS PAGE gels stained by coomassie blue. However, upon incubation of ^3H -EYL sonicated liposomes with high

Figure 1. Elution Profile of α BGT on a Whatman CM-32 Column.

■—■, relative lipase activity; ●—●, optical density at 280 nm. Gradient elution was performed as indicated in Methods.

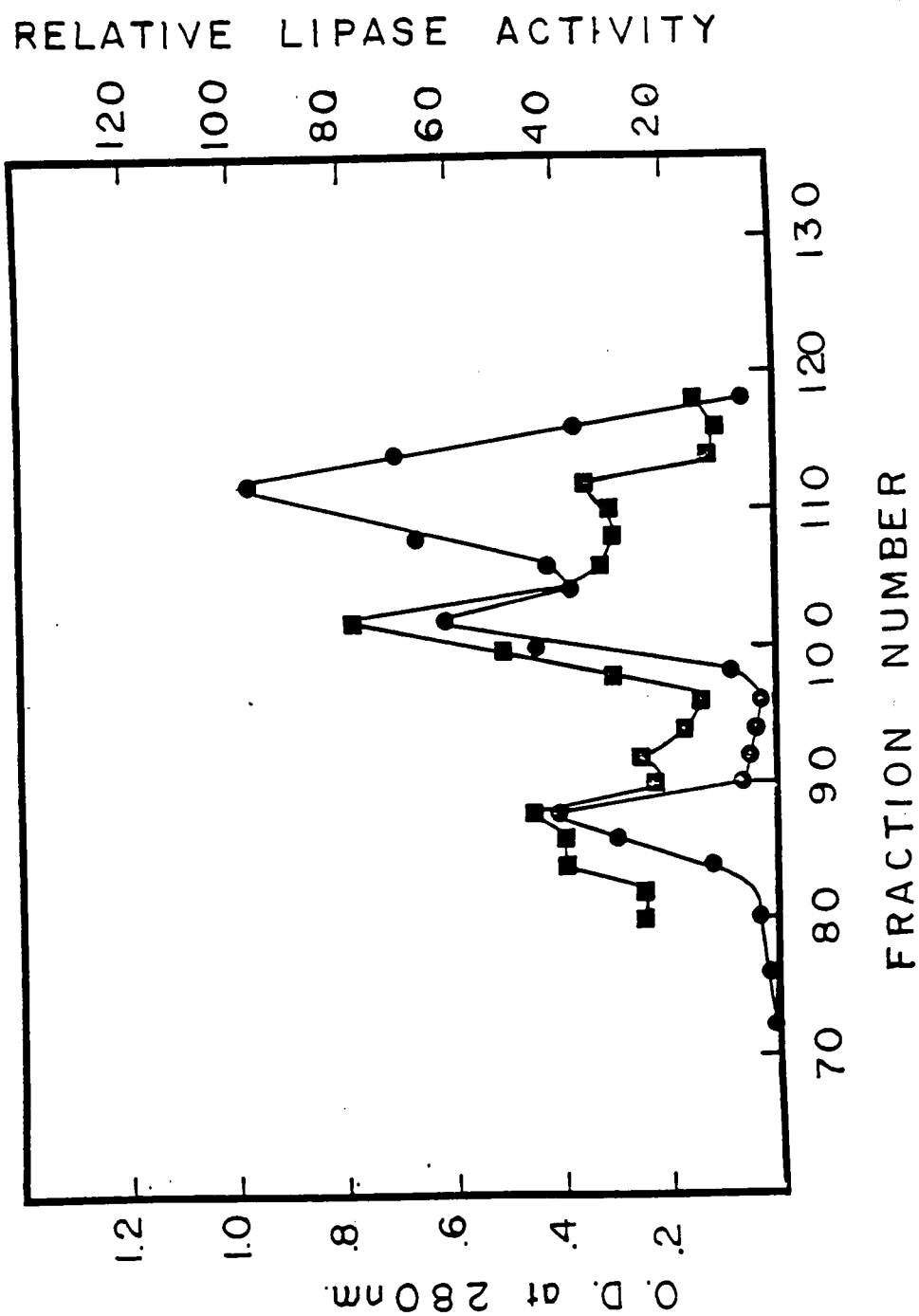


Figure 1

concentrations of α BGT (5.0×10^{-4} M) for one hour at 37 C in PBS a major conversion of the lipid into both free fatty acid and lyso-phosphatidylcholine resulted from contaminating phospholipase A₂ activity. Therefore, routine analysis for phospholipase activity was carried out on all protein fractions on the CM-32 column shown in Figure 1 and only those fractions with greatly reduced phospholipase activity were pooled. It has been shown that Ca^{+2} was required for this enzymatic activity and at concentrations greater than 5 mM EGTA reduced this activity to near basal levels (69-70). Therefore, to all pooled fractions of peak II₂, EGTA was added to a final concentration of 5 mM.

Coupling Reaction of α BGT and Octanoic Acid Anhydride

The anhydrides of octanoic, decanoic, dodecanoic, tetradecanoic and hexadecanoic acids were prepared. Their reactions with ^{125}I - α BGT were attempted. Although all of the anhydrides produced coupled products with ^{125}I - α BGT, only the octanoic acid anhydride reaction produced a satisfactory yield. The anhydrides of longer chain fatty acids caused either an extensive solubility problem or their coupling efficiency was too low to be pursued further. In order to overcome the rapid hydrolysis of the acid anhydride, mole ratios between 100:1 to 1000:1 of anhydride to protein were chosen to obtain effective coupling. Ratios higher than 100:1 caused problems in the purification of the coupling product. A strong buffer capacity (0.3 M phosphate buffer) was necessary to maintain the pH of the reaction mixture as free acid was produced in the reaction.

Purification of Mono-Octanoyl ^{125}I - αBGT

All the reaction products from the conjugation reactions were first separated by Sephadex LH-20 column chromatography. Figure 2 shows a typical elution profile of an experiment in which ^{14}C -octanoic acid anhydride and ^{125}I - αBGT were used as reactants. Satisfactory separation of the excess ^{14}C -octanoic acid from the ^{125}I -toxin was obtained. However, the fatty acid coupled toxin indicated by a small peak (fractions 8-10) of ^{14}C coeluted with ^{125}I , was not separated from the unreacted ^{125}I -BGT. It was necessary to chromatograph it further on a Phenyl-sepharose column (Figure 3). The unreacted toxin was cleanly eluted with PBS. The fatty acid coupled toxin was not eluted until the elution solvent was changed. A peak containing both ^{125}I and ^{14}C radioactivities was eluted in 70% ETOH. The ratio of octanoic acid to αBGT , calculated from the corresponding specific activities, was 1.0 ± 0.1 in four experiments. The material in this peak was assigned as mono-octanoyl- αBGT (OBGT). The yield of the purified OBGT as percent of initial ^{125}I -BGT depends on the ratio of octanoic acid anhydride to ^{125}I -BGT in the reaction mixture. A typical yield of 15-20% was obtained with a ratio of 1000:1. The multiply coupled ^{125}I -BGT was not eluted in 70% ETOH and could be eluted by using more nonpolar solvents such as CHCl_3 . Their yields were too small to warrant further studies.

The evidence for the covalent nature of the coupling between the fatty acid and ^{125}I -BGT in the purified OBGT came from the following results:

1. OBGT was soluble in ETOH at a concentration in the range of 1.0 nmoles/ml, whereas the ^{125}I -BGT was not.

Figure 2. Elution Profile of the OBGT Synthetic Reaction Products on a Sephadex LH-20 Column Eluted with PBS Buffer pH 7.4.
●—●, ^{125}I -BGT; ○—○, ^{14}C -octanoic acid.

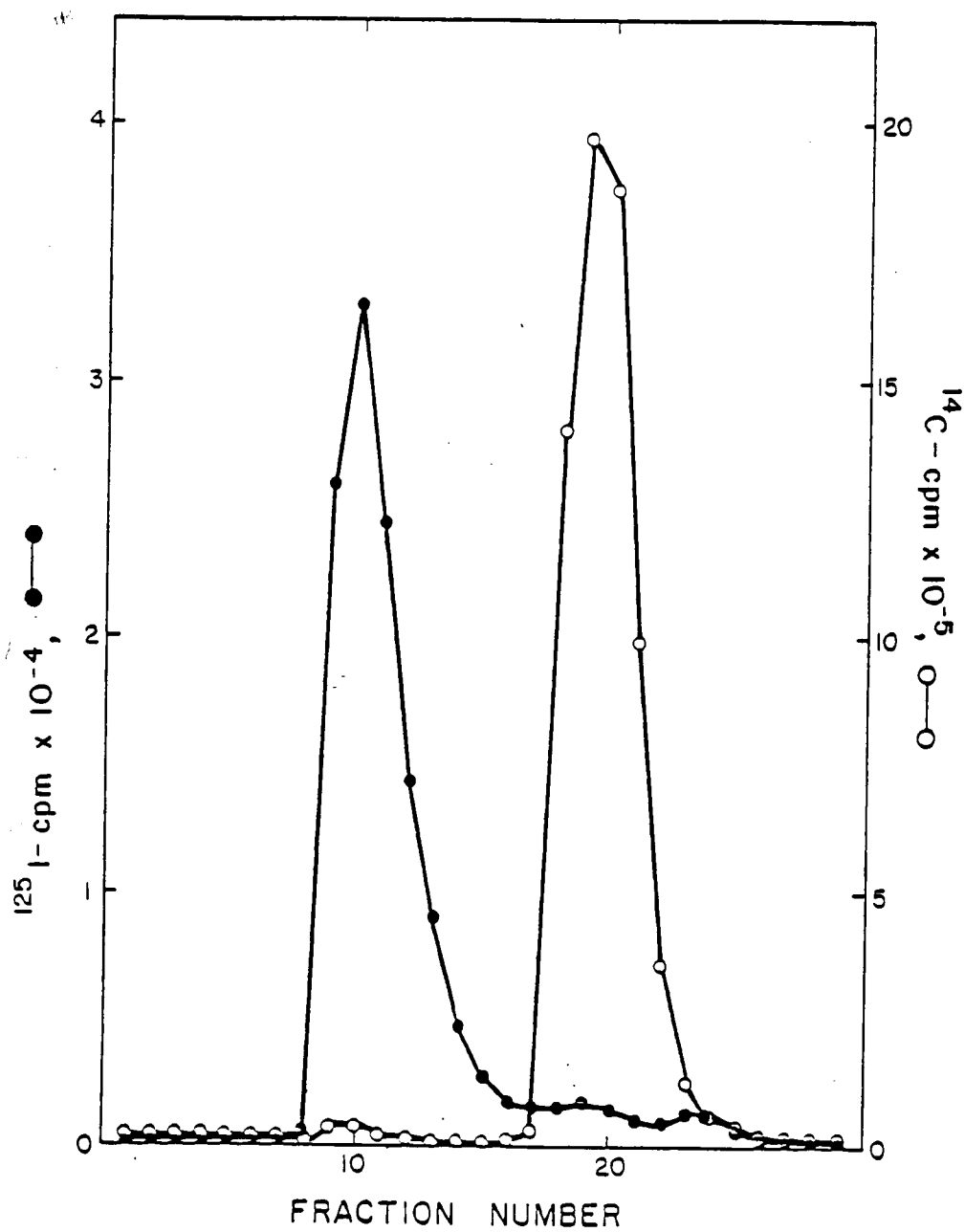


Figure 2

Figure 3. Elution Profile of OBGT and Unreacted α BGT on a Phenyl-Sephadex Column.

Fractions 1-60 were eluted with PBS buffer pH 7.4; fractions 60-100 were eluted with 50% ETOH in PBS. Pooled samples in barred area were labeled OBGT. ●—●, ^{125} I-BGT; ○—○, ^{14} C-octanoic acid. The change in elution buffer was indicated by the arrow.

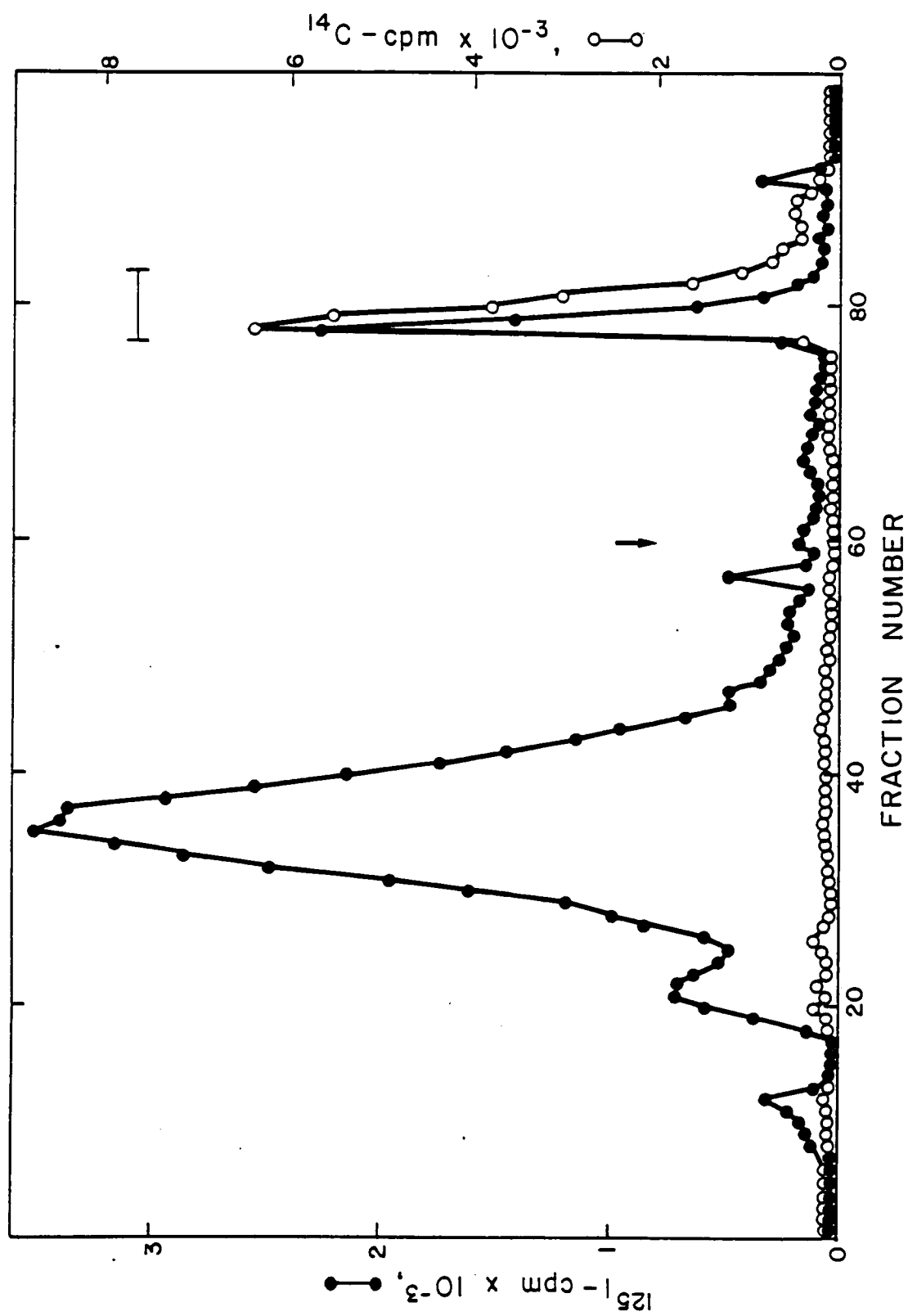


Figure 3

2. The ^{14}C and ^{125}I radioactivities of the OBGT were always partitioned together in the two-phase solvent systems of benzene/water, CHCl_3 /water or toluene/water.
3. The ^{14}C and ^{125}I radioactivities of OBGT appeared as a single band which migrated slightly slower than the ^{125}I -BGT in sodium dodecylsulfate polyacrylamide gel electrophoresis (data not shown).

Binding of OBGT to PC Vesicles

When a suspension of ^{125}I -OBGT was mixed with preformed ^3H -MLV, the binding of ^{125}I -OBGT to the vesicles was measured after the removal of unbound ^{125}I -OBGT by centrifugation and washing. The amount of binding increased linearly with the concentration of added ^{125}I -OBGT (Figure 4a). In control experiments, the underivatized ^{125}I - α BGT only showed a small amount of binding. The data was analyzed in terms of a Scatchard plot (71). The dissociation constant, K_D , of ^{125}I -OBGT to vesicles was about 1×10^{-7} M. Since there was only about 15% of the total vesicle lipid exposed to the outermost bilayer surface in MLV (72-73), the binding would be enhanced if the inner lipid bilayers were also available for binding. This was confirmed by first mixing PC and OBGT in ETOH, then removal of solvent in vacuum, followed by mechanical agitation in PBS to prepare MLV's. Figure 4b shows the result of binding in such an experiment. Indeed, the binding of OBGT to the bilayer lipids in these vesicles increased about 10-fold over those described in Figure 4a. Again, the underivatized ^{125}I -BGT showed minimal binding activity.

Figure 4. Binding of OBGT and α BGT to Lipid Vesicles.

a) Binding to preformed MLV; b) binding induced during the formation of MLV.

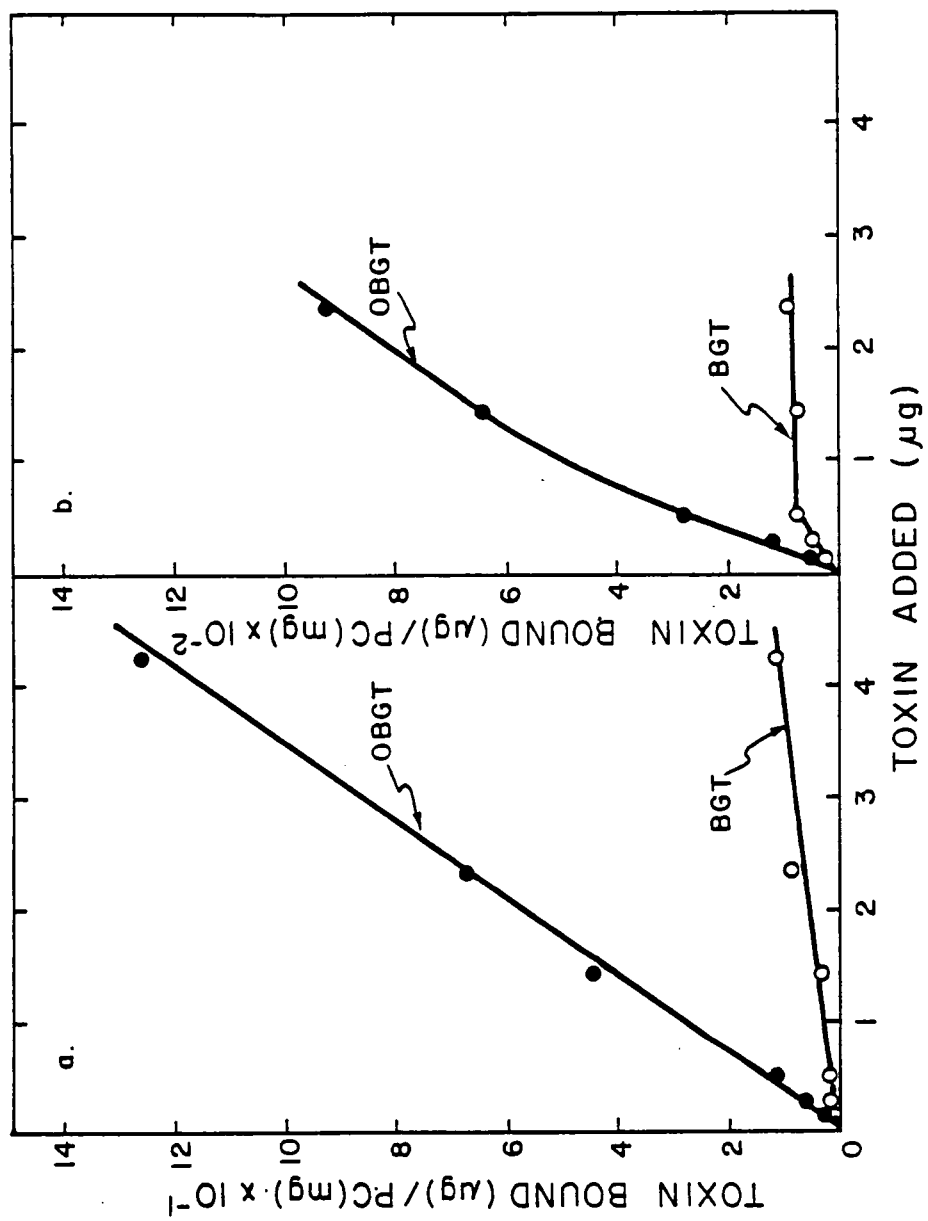


Figure 4

We then investigated to see if ^{125}I -OBGT was able to bind with the sonicated vesicles. Preformed ^3H -vesicles were mixed with ^{125}I -BGT or OBGT. The binding was then measured by Sepharose-4B column chromatography (Figure 5). It was obvious that the underivatized ^{125}I -BGT had little, if any, affinity for the vesicles, whereas the OBGT showed substantial binding under identical conditions. Again, the binding of OBGT to vesicles could be significantly enhanced if OBGT and PC were mixed in ETOH first, and then cosonicated in PBS buffer following solvent removal (data not shown).

Binding of OBGT to Detergent Solubilized AchR

It was important to find out how much of the specific binding activity of ^{125}I -BGT to AchR was still retained after coupling with fatty acid. This was done by adding various unlabeled competitors to the incubation mixture prior to the addition of a saturating amount of ^{125}I -BGT to the triton X-100 solubilized AchR. As shown in Figure 6, the native BGT effectively competed for binding to AchR. The unlabeled OBGT also competed well; a final mole ratio of 1:1 of OBGT to ^{125}I -BGT caused about a 60% inhibition of binding. At ratios up to 10:1, both α -naja toxin and lysozyme did not show any competition for binding. This result was further substantiated by an experiment in which the concentrations of the competing ligands were varied over a large range. The results shown in Figure 7 clearly show that the specific binding affinity of OBGT to AchR was about 10% of that of the native underivatized α BGT. However, the binding capacity of OBGT was still large, at least five orders of magnitude higher than that of α -naja toxin. Therefore, the coupling of a fatty acyl chain to

Figure 5. Binding of OBGT and α BGT for SUV as Measured by Sepharose 4B Column Chromatography.

- Protein and lipid were co-sonicated before column loading.
- a) elution profile for ^{125}I -BGT, ●—● and ^3H -lipids, ○—○;
 - b) elution profile for OBGT, ●—● and ^3H -lipids, ○—○.

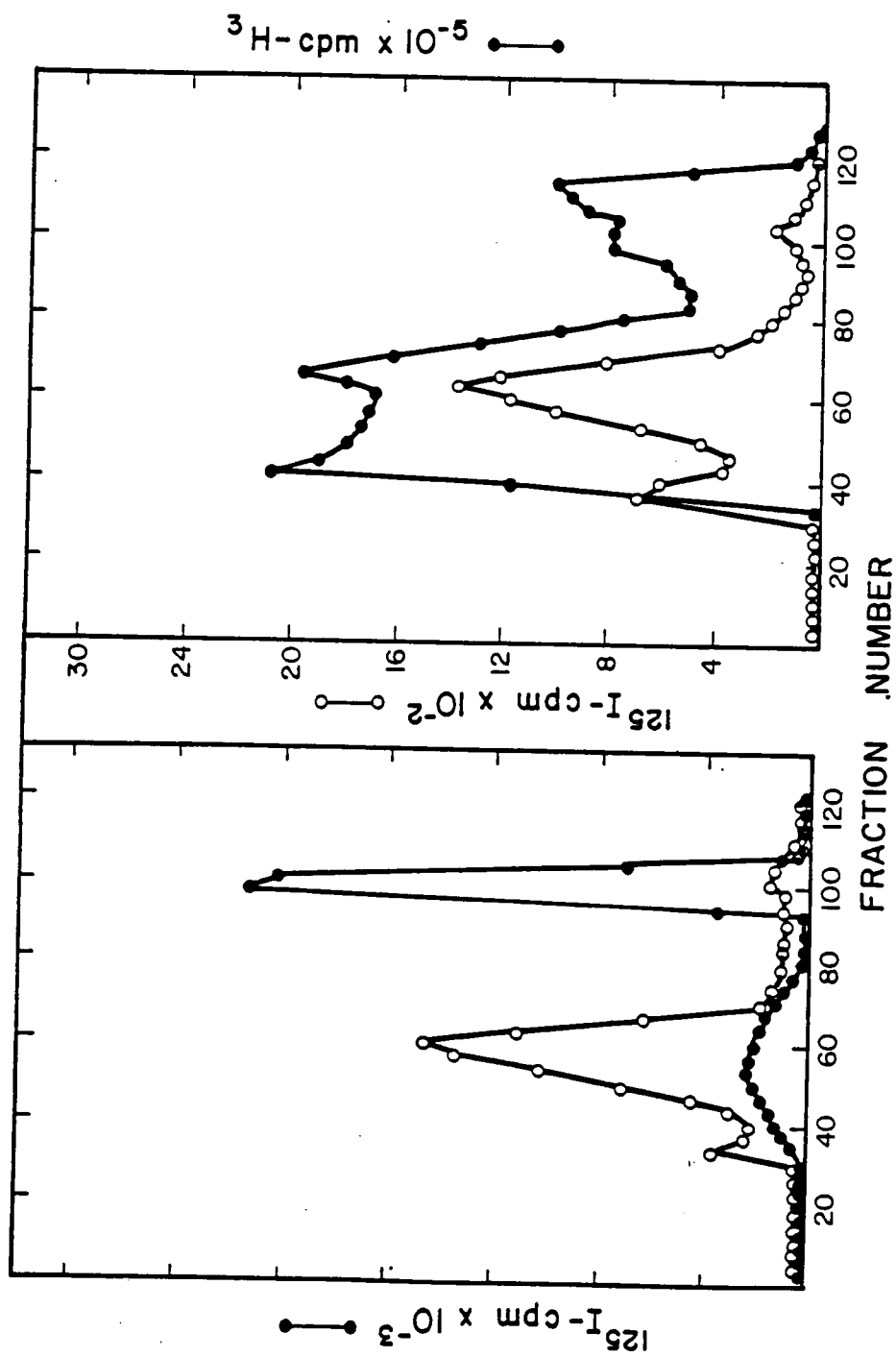


Figure 5

Figure 6. Inhibition of ^{125}I -BGT Binding to Triton Solubilized Receptors by Several Competitors.

○—○, α BGT; ●—●, OBGT; △—△, α -naja toxin; ▲—▲, lysozyme.

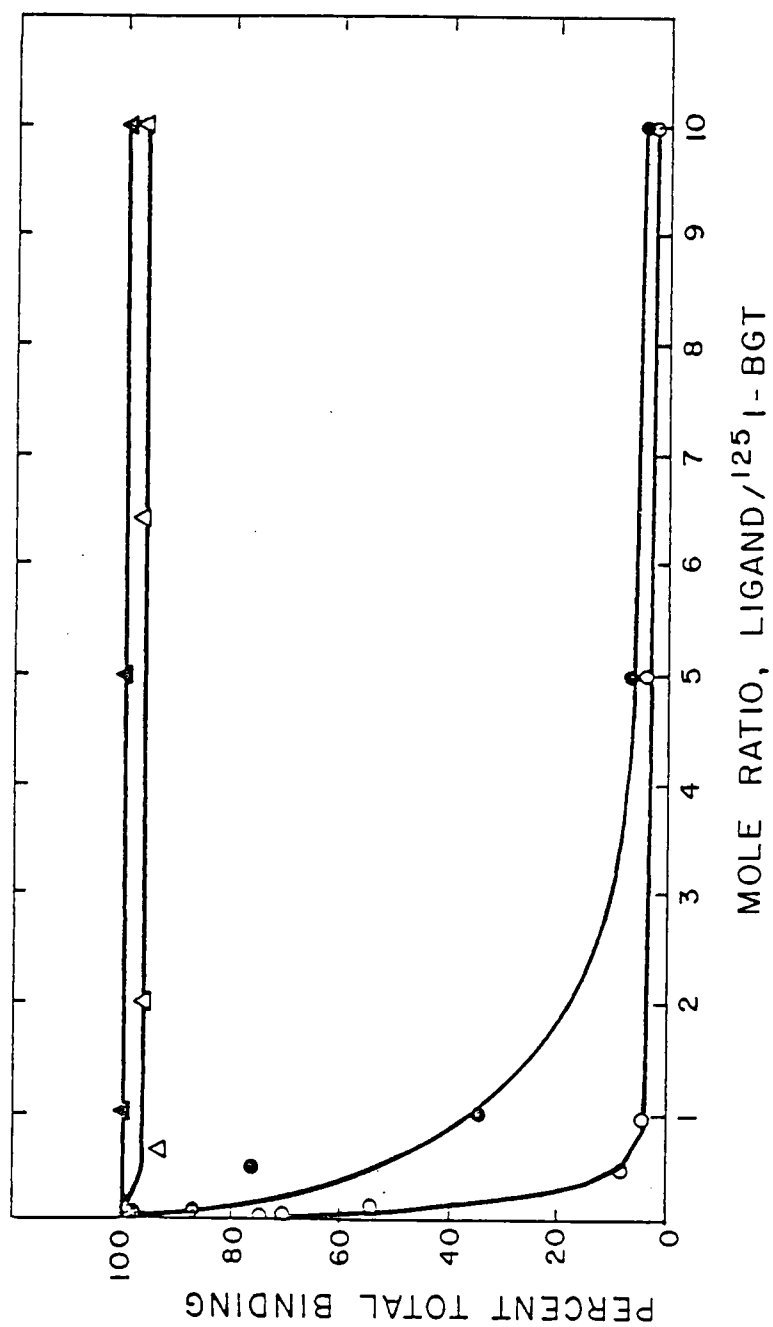


Figure 6

Figure 7. Inhibition of ^{125}I -BGT Binding to Triton Solubilized Torpedo Receptors by Several Competitors.

A wide range of concentration of the competitors was used.
○—○, α BGT; ●—●, OBGT; Δ — Δ , α -najatoxin; $\blacktriangle$ — $\blacktriangle$, lysozyme.

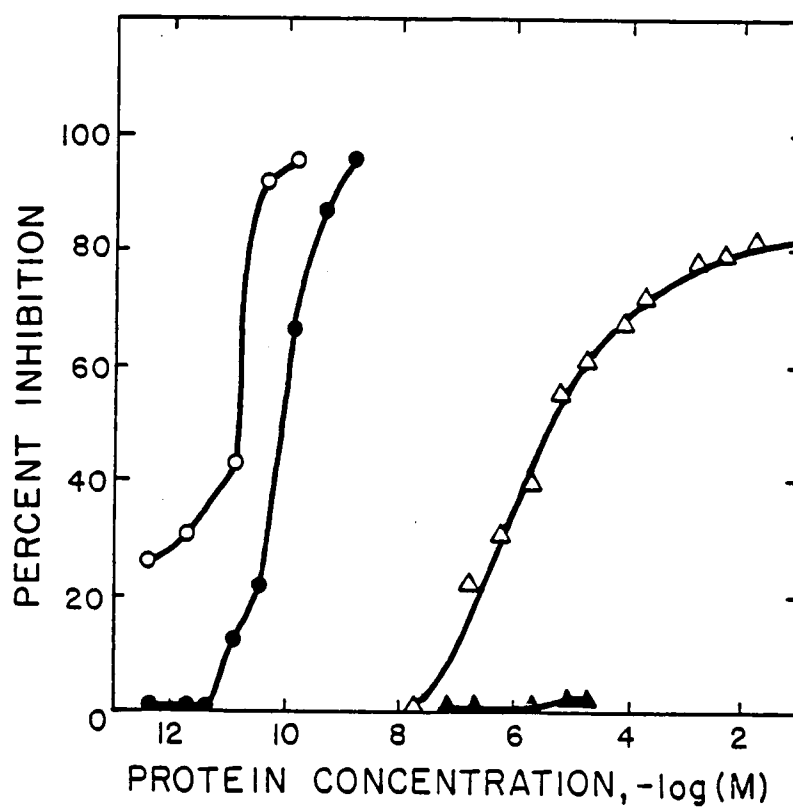


Figure 7

α BGT did reduce the specific binding affinity of the toxin to AchR, but OBGT retained sufficient binding affinity for liposome targeting. This binding affinity was in the range of affinities for antigen-antibody interaction.

OBGT Vesicle Association with Microsacs

Table 1 shows that OBGT vesicles have a large affinity (about 35% associated tritium counts) for microsacs when compared to control membranes of RBC ghosts (<10% associated tritium counts). There was also a significant increase in the percentage of ^{125}I counts associated with microsacs compared to control membrane. It was apparent that there was a small percentage (5%) of tritium counts which was non-specific association. It was apparent that the amount of OBGT binding to either microsacs or RBC ghosts was consistently higher than that of the lipid binding.

Discussion

The purification of α BGT from crude venom produced elution profiles similar to those reported by C.Y. Lee (73). The protein was identified as a single band on SDS-PAGE in both slab and tube gels. Based upon known molecular weight of internal protein standards, this band has an estimated weight of slightly greater than 8,000 daltons. There was an active phospholipase A_2 activity associated with this material. It was possible that a small ($\leq 1.0\%$) amount of contaminating β BGT, a potent phospholipase A_2 , was responsible for this activity. However, as discussed in Materials and Methods

TABLE 1

BINDING OF DOL VESICLES TO MEMBRANES^a

Membrane ^b	Vesicle Containing OBGT ^{c,d}	³ H-DOL		¹²⁵ I-OBGT	
		cpm bound	% added	cpm bound	% added
Microsacs	No	11,250	15	--	--
Microsacs	Yes	25,680	35	49,976	41
RBC ghost	Yes	6,530	9	32,829	27
RBC ghost	No	3,600	5	--	--

^aIncubation was 30 minutes at 4°C.

^bTotal membrane protein was 0.1 mg in all cases.

^cTotal ³H- and ¹²⁵I-cpm of OBGT-vesicles added were 73,160 and 121,856, respectively; total ³H-cpm of non-targeted vesicles added was 65,530.

^dOBGT to vesicle ratio is 6.6.

(page 13), the addition of EGTA to remove calcium from the buffer diminished this lipase activity below detectable levels. It was important to remove even trace activity of the contaminating phospholipase since it could destroy the vesicle integrity by hydrolyzing the phospholipids.

The coupling of octanoic acid to α BGT changed the toxin molecule into an hydrophobic molecule. This protein demonstrated many characteristics of an hydrophobic membrane protein. In PBS buffer, the protein tended to aggregate into larger micelles. At a concentration of 0.1 mg/ml, the protein in solution was a suspension. A small amount of detergent (<0.01%) would convert the suspension into a clear solution. It was evident that OBGT would partition into the more hydrophobic phase of a two-phase solvent system. In the presence of liposomes, as shown in Figures 4 and 5 (pages 29 and 32), OBGT associated with liposomes. This interaction was a stable one, since the OBGT-vesicles could be maintained for more than 48 hours without significant loss of protein.

The modified toxins were incorporated into lipid vesicles and the resulting vesicles were tested for binding with the microsac membranes enriched with the acetylcholine receptors. The results in Table 1 suggest that OBGT may enhance (two-fold) the binding of vesicles to the microsacs when compared to the protein-free vesicles. However, this enhancement in binding was overshadowed by a similar increased association of OBGT vesicles with RBC ghosts, even though the binding to the control membranes remained at a low level. An OBGT-like ligand may be useful as a targeting molecule for liposomes in this model system.

It is also apparent in Table 1 that the binding of toxin to either microsacs or RBC ghosts was higher than the concomitant binding of lipids for OBGT-vesicles. The discrepancy may be explained when one examines the relative affinities of OBGT to bind with the vesicles and receptors. The dissociation constant, K_D , of OBGT to lipid vesicles has been estimated to be 1×10^{-7} M (Figure 5, page 32). This affinity was about one order of magnitude lower than the binding of OBGT to receptors. OBGT had a K_D for detergent-solubilized receptors around 10^{-8} M as measured by an indirect competition assay (Figures 6 and 7). Under conditions in which some stress forces were large enough to disrupt the association of the liposomes with the microsacs, it was likely that some OBGT would be unrooted from the lipid bilayer before the breaking of the ligand-receptor bond. In other words, liposomes may have dissociated and left some OBGT with the microsacs. The result was a higher binding of OBGT than lipids. This may have been a direct consequence of a relatively weak anchorage of OBGT into the bilayers of the vesicles by using a short chain fatty acid of only eight carbon atoms.

The concept of multivalent binding was not examined with OBGT. The relatively poor yield of OBGT synthesis and purification has prevented us from incorporating larger amounts of toxin into vesicles than those used in experiments described in Table 1. As discussed in Chapter I (page 8), multivalent binding could only take place when sufficient toxin molecules were bound to the vesicle surface. It was clear that an alternative method of derivatization had to be used. It was for this reason and the one described above for the need of longer acyl chain anchorage that the use of the N-hydroxy

succinimide ester of fatty acid to prepare fatty acid derivatives of α BGT. This is described in the next chapter.

The primary goals in the characterization of the purified OBGT were to determine if this product still retained its primary biological functions and gained an affinity for a lipid environment. Both of these properties were necessary if OBGT was to be a good representative model of targeting molecules.

The affinity of OBGT for a lipid environment is shown in Figures 5, 6 and 7 (pages 32, 34 and 36). The covalent modification of this protein dramatically changed its physical properties such that OBGT truly reflected a protein whose properties were more representative of hydrophobic proteins. Several changes in the physical properties of the fatty acid-protein conjugate were immediately apparent following its purification and resuspension in PBS buffer. Upon resuspension, OBGT tended to aggregate. The addition of a small amount of detergent or the addition of high levels of free octanoic acid, dissolved these aggregates and produced a true solution. OBGT would partition into any lipid membrane environment to which it comes in contact. This characteristic made impossible direct binding assays to microsacs in the absence of detergent.

CHAPTER III

STUDIES WITH MONO-PALMITOYL- α -BUNGAROTOXIN

Abstract

We have covalently coupled palmitic acid to α -bungarotoxin (α BGT). The coupling reaction was carried out by using the N-hydroxysuccinimide ester of palmitic acid in the presence of deoxycholate (DOC). The reaction mixture was fractionated by hydrophobic column chromatography with Sephadex LH-20 and phenyl-Sepharose. The fatty acid to protein mole ratio of the purified product (overall yield >60%) was approximately one. This product, mono-palmitoyl- α BGT (PBGT), effectively and specifically inhibited the binding of ^{125}I - α BGT to the triton-solubilized acetylcholine receptors from I. californica. PBGT aggregated at $[\text{DOC}] < 0.1\%$ (w/v). Isoelectric focusing PAGE indicated that the palmitic acid was covalently coupled to α BGT, probably through a basic amino acid residue. PBGT, in contrast to the native α BGT, bound effectively to preformed liposomes. Greater than 90% of PBGT was incorporated into the unilamellar vesicles (500-1000 $\text{\AA}$ in diameter) when a DOC-solubilized solution of PBGT and dioleoyl lecithin was chromatographed on Sepharose 4B. Furthermore, PBGT-vesicles showed a specific binding to the Torpedo microsac membranes but not to red blood cell ghosts. The binding of these vesicles with microsac membranes requires a threshold ratio of PBGT to phospholipid in vesicles, suggesting a positive cooperativity in binding. This

system is not only a good model for studies on liposome targeting, but is also valuable in studies of membrane-membrane interactions mediated through specific ligand-receptor binding.

Introduction

In the preceding chapter, I have described the synthesis and characterization of the mono-octanoyl- α BGT (OBGT). Although OBGT can be used as a specific ligand coupled to liposomes and the resulting liposomes show some enhanced binding to the target membrane, i.e. the microsacs of electric fish, a number of problems in using OBGT as a targeting molecule become apparent. Firstly, the yield of OBGT in the synthesis and purification steps is too low to put enough targeting molecules on the liposomes for a multivalent binding between liposomes and their target membrane to occur. Secondly, the fatty acyl chain length of OBGT is probably too short to stably anchor the targeting molecule on the liposomal membrane.

In view of these potential drawbacks, I have decided to explore alternative methods to conjugate a longer fatty acyl chain to α BGT with high yield. In this chapter, a new method using the N-hydroxysuccinimide ester of palmitic acid is described. This improved method gave a much higher yield of the conjugated α BGT. The fatty acyl chain length has increased from eight to 16 carbons. The characterization of the palmitic acid conjugated α BGT and its use in liposome targeting are also described.

Materials and Methods

Preparation of Microsac Membranes

Microsac membranes were prepared essentially as described in the preceding chapter with the following modifications: Phenylmethyl sulfonyl fluoride (PMSF) was included (5.0 mM) in the homogenization buffer and resuspension buffers to inhibit the protease activity. Membranes were resuspended by Dounce homogenization (40-50 strokes), and the low speed spin was increased to 12,000 x g for 15 minutes. This step was repeated once.

Preparation of the N-hydroxysuccinimide Ester of Palmitic Acid

We used a modification of the general synthetic procedure described by Y. Lapidot, S. Rappoport and Y. Wolman in a report on the use of similar activated esters in the synthesis of N-acylamino acids (73). In our modification, the recrystallization was carried out twice from anhydrous ethanol (redistilled ethanol over sodium and stored in molecular sieves). The by-product of the recrystallization was removed by vacuum filtration on a Buchler funnel 50 ml capacity. Finally, the product was redissolved in anhydrous (refluxed over sodium for two hours) 1,4-dioxane at a concentration of 0.119 M. This solution was stored under dry N₂ and dessicated.

Covalent Coupling of Palmitic Acid to α BGT

The activated palmitic acid ester of succinic acid (0.714 μ moles in 6 μ l of anhydrous 1,4 dioxane) was delivered to a clean dry 12 x 75 mm test tube and the solvent removed by a dry stream of N₂ gas.

This dry film of activated ester was reacted with ^{125}I -BGT (0.238 μmoles) in 0.5 ml of PBS buffer pH 7.4 containing 2.0% DOC. These conditions gave a three-fold excess of coupling reagent compared to protein. This reaction was initiated first by mild bath sonication for one minute which broke up the insoluble coupling reagent into small aggregates forming a suspension. The reaction mixture was heated in a water bath for one hour at 37°C. The suspension turned into a true solution after 10 minutes at the elevated temperature. Following the incubation, the reaction was diluted to a DOC concentration of 0.5% by the addition of 3.5 ml of PBS buffer, pH 7.4. The purification of the reaction product was accomplished using hydrophobic gel chromatography.

Purification of PBGT

The purification of PBGT used the same protocol as was used in the purification of OBGT. This technique used the two hydrophobic gels Sephadex LH-20 and phenyl-Sepharose. The only difference in the procedure is the fact that the sample was layered onto the Sephadex LH-20 column in the presence of 0.5% DOC. The detergent was retarded by the interaction with the gel. Therefore, PBGT was effectively removed from the detergent. Apparently, the free palmitic acid not coupled to protein was retarded by the gel matrix.

Preparation of Large Unilamellar Phospholipid Vesicles

The preparation of large unilamellar vesicles (LUV's) was carried out essentially as described by Enoch and Strittmatter using the detergent deoxycholate (DOC), followed by gel filtration to remove

DOC (74). There were three modifications added to the original technique. Firstly, the lipid used was dioleoyl phosphatidylcholine containing trace ^3H -hexadecylcholesterol such that the final specific activity with respect to the DOL is 1.0×10^{12} cpm/mole and the final concentration of total lipid is 10 mg/ml. ^3H -hexadecylcholesterol has been shown by this laboratory to be a faithful non-exchangeable, non-degradable lipid bilayer marker (75). Secondly, slightly higher mole ratios of DOC to lipid (1.5:2) were used when PBGT was present because of the highly lipophilic nature of this protein. Finally, after the gel filtration on Sepharose 4B or Sephadex G-50 (fine) column (1 x 53 cm), it was necessary to dialyze in ultra thin-walled dialysis tubing, Spectrapor 2 (Spectrum Medical Industries, Inc., Los Angeles) against PBS buffer pH 7.4 for 72 hours with six buffer exchanges. The residual insoluble DOC after dialysis was pelleted by low speed centrifugation (300 rpm x 5 minutes, Sorvall GLC-2). The supernatant contained 75% of both protein and lipid originally placed on the gel filtration column.

Preparation of Triton-solubilized Torpedo Acetylcholine Receptor

The isolation of a partially purified Triton X-100 solubilized acetylcholine receptor from the electric organ of Torpedo californica was necessary to do direct binding assays. The following method was used which is a minor modification of the isolation procedure described in the preceding chapter.

Seventy gms of frozen tissue from the electric organ of Torpedo californica were thawed, cut and minced into small pieces. Thirty ml

of buffer A (0.2 M NaCl, 10 mM Tris pH 7.8, 5 mM EDTA 0.02% NaN₃ and 5 mM PMSF) was then added and complete thawing was accomplished. The solution was homogenized for one minute in an Omni mixer at maximum speed. The homogenate was sieved through a polypropylene screen (420 mesh, 34% open area) to remove connective tissue, then sieved through 4-ply gauze to remove small fibrous material. The solution was then diluted with 25 ml of buffer A and centrifuged 30 minutes at 30 k rpm (100,000 x g) in a type 30 Beckman rotor. The supernatant was removed and the pellet was washed (3X) with 2 ml of buffer A. The pellet was then resuspended with 60 ml of buffer B (1 M NaCl, 10 mM Tris pH 7.8, 7 mM EDTA 0.02% NaN₃ and 5 mM PMSF) by vortexing and then homogenized with a ground glass homogenizer for 20 strokes. The resuspended solution was again centrifuged as above. The supernatant was removed and the pellet washed (3X) with 2 ml of buffer B. Pellets were pooled and extracted with a solution containing 2% Triton X-100, 100 mM NaCl, 10 mM Tris pH 7.8, 5 mM EDTA, 0.02% NaN₃ and 5 mM PMSF for two hours at room temperature with constant rotor for 60 minutes. The supernatant was concentrated in an Aminco concentrator using XM 50 filter at 30 psi. The concentrated solution (2.5 ml) was mixed with 2.5 gms SM-2 beads (BioRad) for two hours to absorb most excess detergent. The sample was frozen at -20°C overnight and bath sonified (Bronson model B sonifier) for two minutes in a 12 x 75 mm glass test tube. The sample was then centrifuged at 3000 rpm in a Sorvall GLC for five minutes and the supernatant was diluted to a final protein concentration of 10 mg/ml. This solution was called Triton-solubilized Torpedo receptor (TSTR) solution.

Competition Assay of ^{125}I -bungarotoxin Binding to Triton-solubilized Torpedo Receptor (TSTR)

A sample (5.0 μl) of TSTR (1.0 $\mu\text{g}/\mu\text{l}$) was placed in a small 12 x 75 mm culture tube. An appropriate amount (10 μg - 100 μg protein) of the competitor was then added and the volume was adjusted to 0.2 ml with Tris-triton buffer (T/T buffer) which contained 2.0% w/v Triton X-100 in 10.0 mM Tris pH 7.8. This solution was incubated 30 minutes at 37°C. A saturating amount (20 μl of ^{125}I -BGT (2.5 x 10⁻⁴ M, specific activity 1.0 x 10¹⁴ cpm/mole) was added to each tube, vortexed and incubated at 37°C for 30 minutes. A 100 μl aliquot from each tube was applied to a stack of ion exchange filter papers in which a CM-81 filter (2.5 cm in diameter) was on top of three DE-81 filters. This entire sandwich of filters was pre-soaked in 0.4 M sodium phosphate buffer pH 5.7 (P-buffer). After the aliquot soaked into the exchange filters (30 seconds), the filters were rinsed with 0.5 ml of P-buffer and allowed to drain for 30 seconds more. The CM filter was removed and discarded. The three DE-81 filters were placed into a 20 ml scintillation vial and soaked twice for one hour in 15 ml of P-buffer containing 2% Triton, until the background on the control filter (no TSTR applied) was about 2% of the total counts applied. The final rinse was in distilled H₂O. The filters were counted in a gamma counter.

Binding of PBGT to Solubilized Acetylcholine Receptor

Triton X-100 solubilized acetylcholine receptor from Torpedo Californica 5.0 μg of protein in 50 μl of buffer A (100 mM NaCl,

10 mM MOPS, pH 7.4, 0.2% Triton X-100) which contains 100 μ g/ml BSA was added to various concentrations of PBGT in buffer A to a final volume of 150 μ l. Samples were incubated at 24°C for 30 minutes. The samples were diluted to 5 ml with buffer B (10 mM NaCl, 10 mM MOPS pH 7.4, and 0.2% Triton X-100). This solution was then filtered over prewashed DE-81 Whatman 2.5 cm filters (prewashed with 4 ml of buffer B). Filtration was done at low vacuum (about 50 mm Hg) through two discs of DE-81. A sample tube was washed two times with 5 ml of buffer B and then filtered over the DE-81 discs. The filters were finally washed twice with 10 ml of buffer B. Background was determined with no receptor present and triton solubilized receptor saturated with native non-radiolabeled α BGT. Samples were counted by gamma emission using the Beckman Bio-gamma II. This assay was essentially the same as reported by Damle and Karlin (76) (Figure 8).

Binding of Vesicles with Microsac Membranes or RBC Ghosts

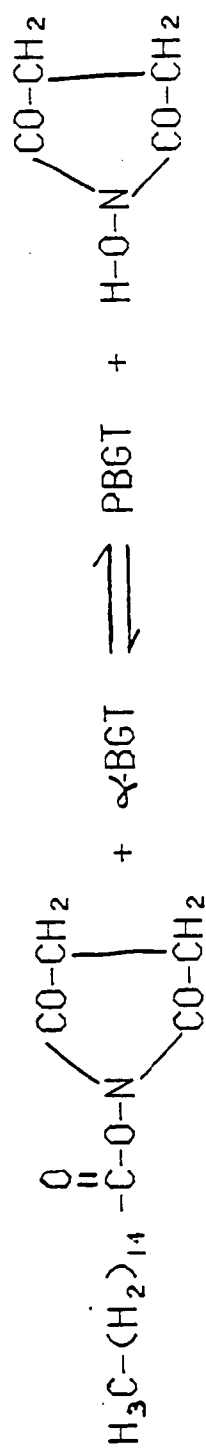
Binding of vesicles with microsac membranes or RBC ghosts was done as described in the preceding chapter.

Results

Purification of Mono-palmitoyl- α BGT (PBGT) (Figure 9)

The elution profile for both isotopes, ^{125} I for the protein and ^{3} H for the palmitic acid formed coincident peaks in the void volume of the Sephadex LH-20 column. Further elution with PBS did not produce any other radioactive peaks, indicating that the excess ^{3} H-palmitic acid was retained in the column. If the pooled peak fractions of the

Figure 8. The Chemical Reaction of the Synthesis of PBGT Using the Coupling Reagent NHSP.



THE SYNTHESIS OF MONO-PALMITOYL-BUNGAROTOXIN (PBGT) USING NHSP

Figure 8

Figure 9. The Purification of PBGT Using Hydrophobic Gel Chromatography.

a) Elution profile of the reaction products on a Sephadex LH-20 column; b) Elution profile of pooled fractions of Sephadex LH-20 column on a phenyl-Sepharose column. ○---○, ^{125}I -BGT; ●---●, ^3H -palmitic acid.

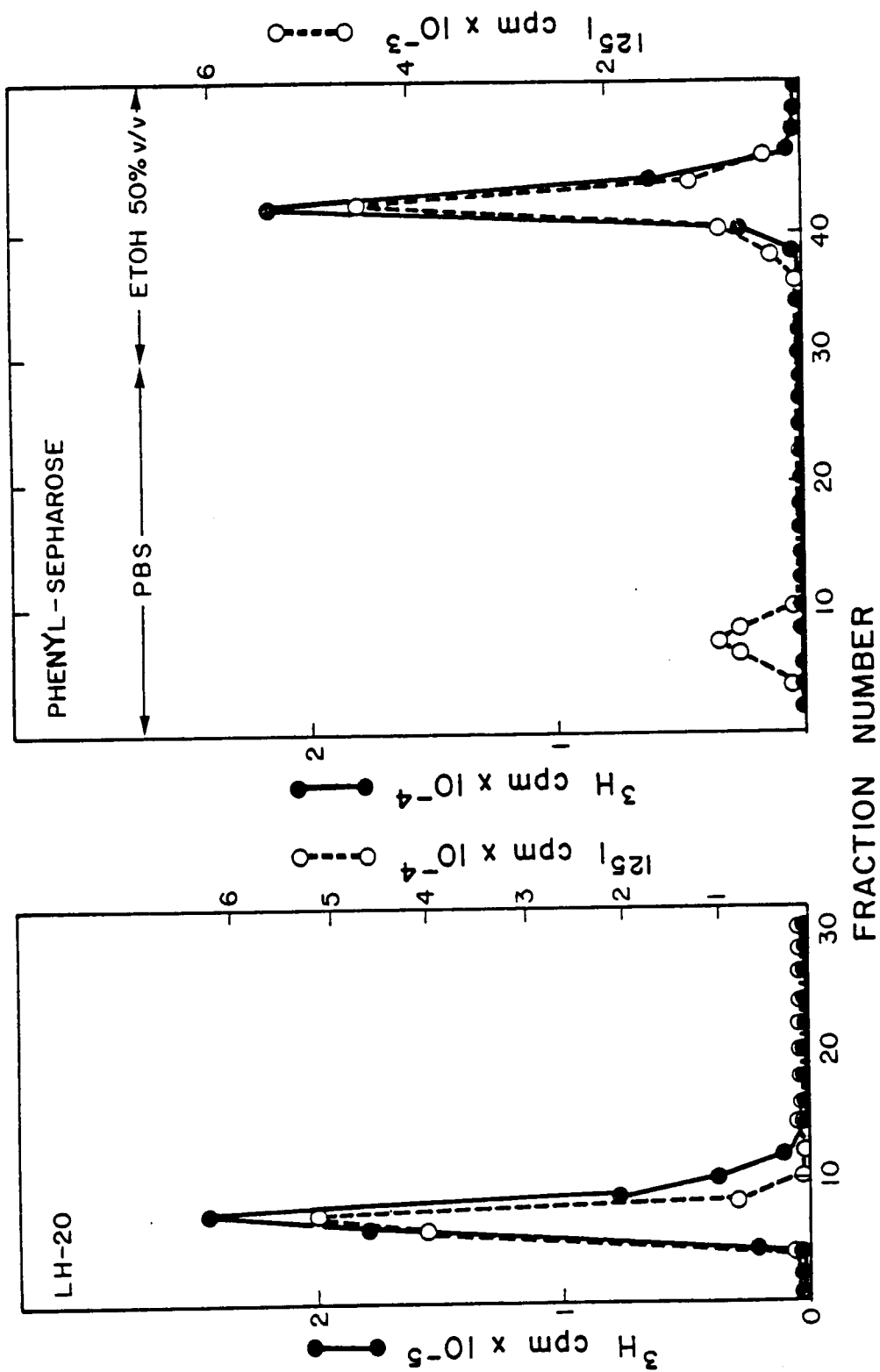


Figure 9

LH-20 elution profile were layered onto a Phenyl-sepharose column which was eluted with PBS buffer, a small ^{125}I radioactive peak was eluted. This small peak which was about 15% of the total loaded onto the column represents unreacted ^{125}I -BGT. No tritium isotope could be found in this peak. Following extensive PBS buffer elution and then eluting with a 50% V/V EtOH:PBS buffer system a peak of the radioactivity containing both isotopes was obtained. The average molar ratio of the isotopes was approximately 1.0. Although this material probably contained a mixed population of fatty acid-protein conjugates, the majority was the mono-derivatized product. Recovery of protein from LH-20 and Phenyl-sepharose was greater than 98% and 80%, respectively. The overall recovery of protein in the form of derivatized product (PBGT) represented 60% of the total starting material. It showed as a single protein band on SDS-PAGE (15% gel) which migrated slightly behind and slightly more diffused as compared to ^{125}I - α BGT. PBGT was not very soluble in phosphate buffer. It aggregated in the absence of a small amount of detergent (<0.01%).

A series of experiments utilizing the double radiolabel techniques in varying concentrations revealed that the molar ratio of palmitic acid to bungarotoxin could be varied from 0.05 to 8.5 when the concentration of ^3H -NHSP varied from 0.1 to 50 times that of bungarotoxin in the reaction mixture. Only preparations of mostly mono-derivatized bungarotoxin were used throughout this work.

Binding of PBGT to Acetylcholine Receptors

The binding of α BGT and PBGT is shown in Figure 10A. Both ^{125}I -BGT and PBGT displayed typical saturation binding in the pmole

Figure 10. Direct Binding Assay of PBGT and α BGT to the Acetylcholine Receptor in Detergent.

a) Equilibrium binding curves; b) Scatchard plots of data from a). K_D for PBGT and α BGT are 2.06×10^{-7} M and 1.16×10^{-8} M, respectively.

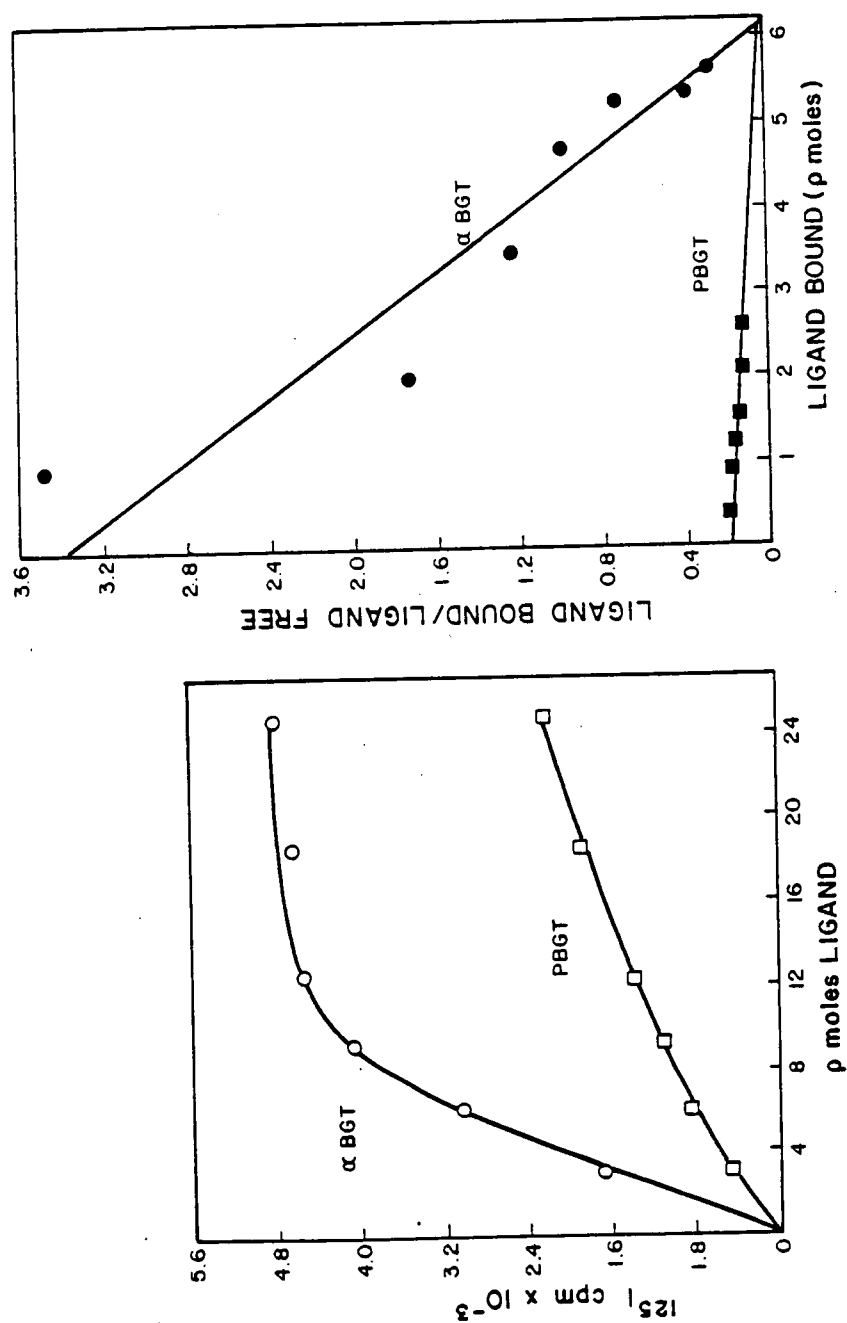


Figure 10

range of the assay. It was quite evident that ^{125}I -BGT had a greater affinity for the acetylcholine receptor than did PBGT. Receptor saturation was achieved at about 10 pmoles for ^{125}I -BGT at which concentration PBGT was far from saturating.

When the data was subjected to Scatchard analysis by the method of least squares fit (77), the dissociation constant (K_D) for ^{125}I -BGT and PBGT was 1.16×10^{-8} M and 2.06×10^{-7} M, respectively (shown in Figure 10b). This K_D value was in the acceptable range 10^{-10} - 10^{-8} M of K_D values published for the binding of ^{125}I -BGT (78-79) to the acetylcholine receptor of Torpedo californica. Moreover, both PBGT and ^{125}I -BGT yielded the same number of receptor sites available for binding (identical intercepts on the x-axis) which strongly suggested that these two ligands with different affinities were binding to the same class of receptor sites.

α BGT and PBGT were further analyzed in terms of their abilities to compete with the binding of ^{125}I -BGT to TSTR. The concentration of PBGT that produced 50% inhibition was about 10-fold higher than that of the α BGT to produce the same amount of inhibition (Figure 11).

We concluded that the binding affinity of PBGT to TSTR was about 10-fold less than α BGT. This was in full agreement with the result of the direct binding assay shown in Figure 10.

Incorporation of PBGT into Unilamellar Lipid Vesicles

Figure 12 shows the result of an experiment which demonstrated the hydrophobic nature of PBGT and its affinity for lipid vesicles. Varying concentrations of PBGT were solubilized using the detergent DOC with a fixed amount of tritiated dioleoyl-lecthin (^3H -DOL), followed

Figure 11. A Competition Assay for Binding of PBGT and α BGT to Detergent-Solubilized Acetylcholine Receptors.

The binding of receptor with ^{125}I - α BGT is competed by various amounts of PBGT or α BGT.

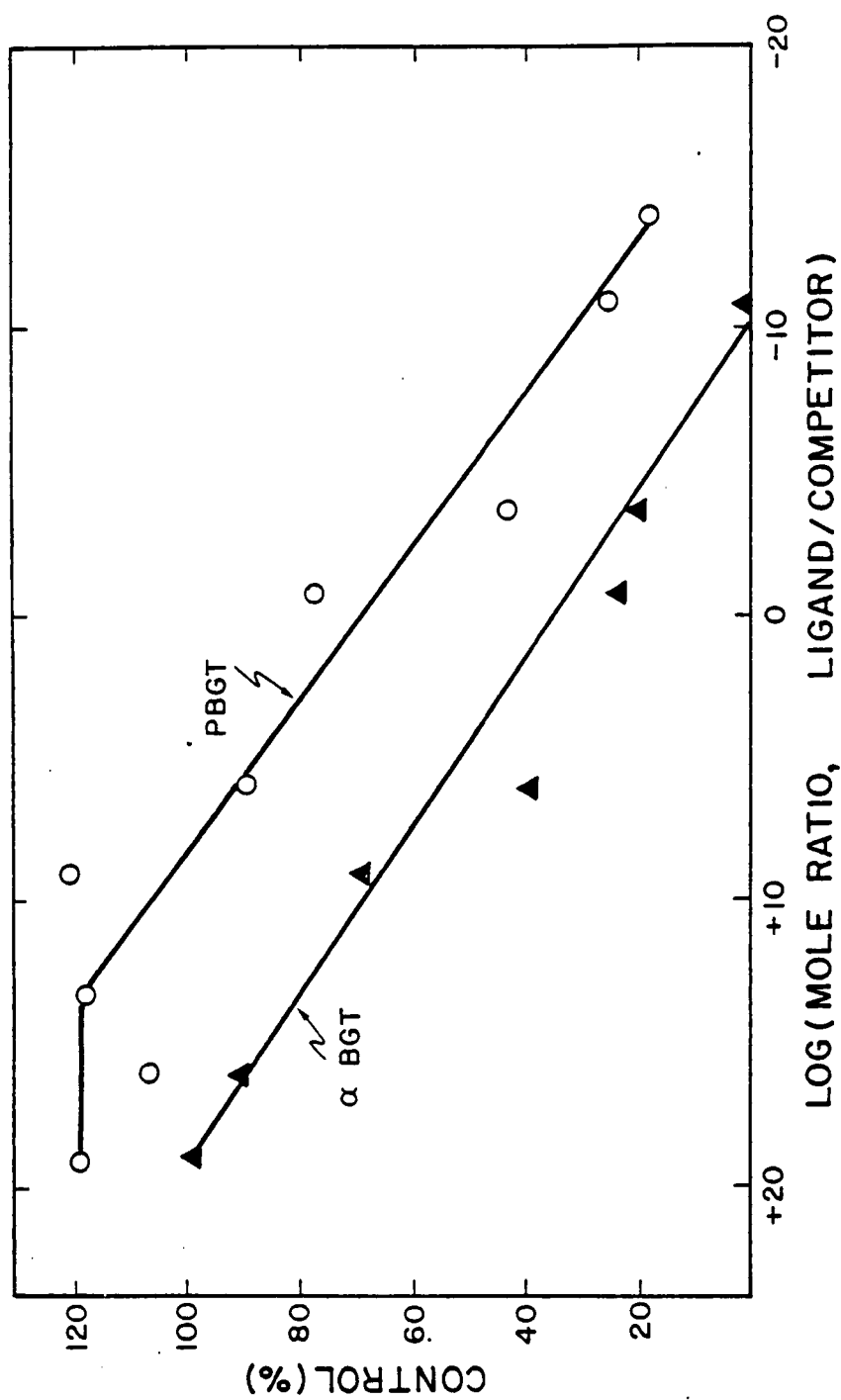


Figure 11

Figure 12. PBGT Association with Lipid Vesicles as Determined by Sepharose 4B Gel Filtration.

●—●, ^3H -DOL; ○---○, PBGT.

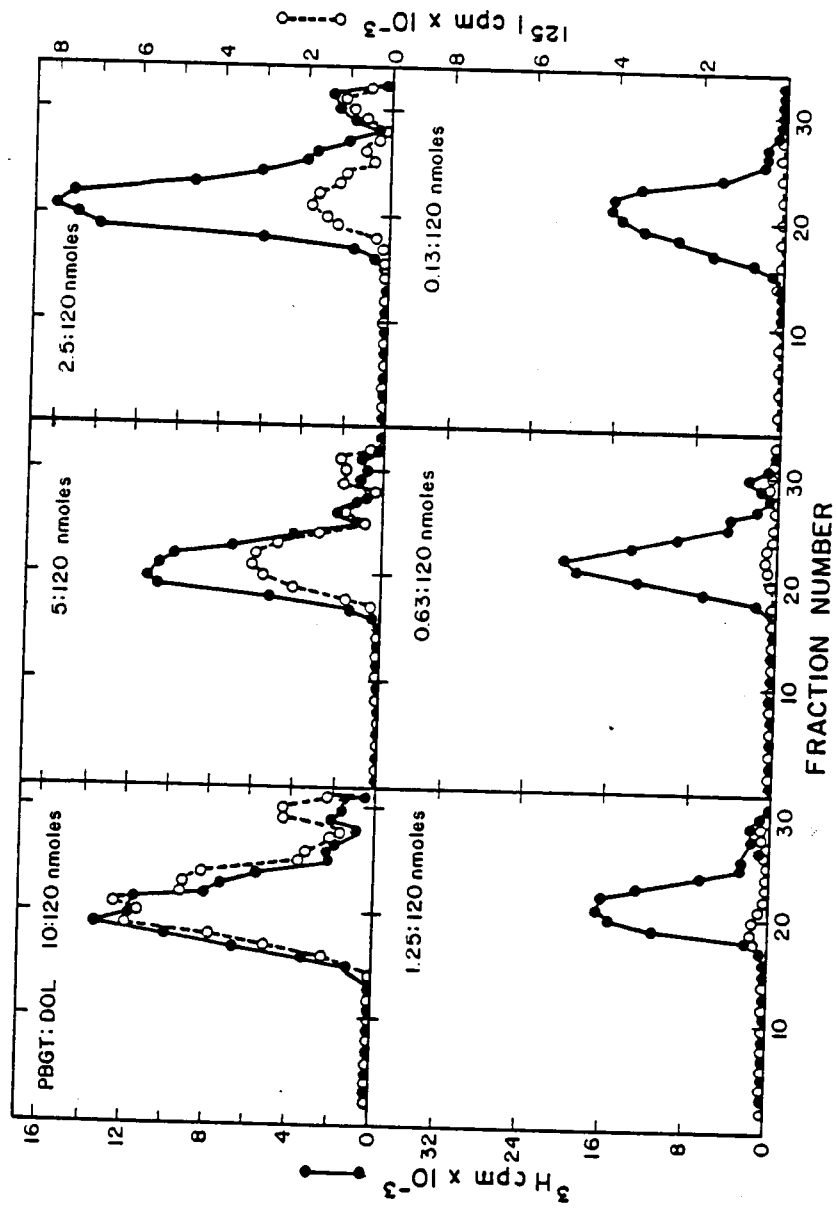


Figure 12

by the removal of DOC with Sepharose 4B column chromatography. The amount of PBGT associated with the liposome peak of the Sepharose-4B elution profile increased with increasing amounts of PBGT. Recovery of PBGT in the Void peak was greater than 80% at all concentrations. However, the recovery of ^3H -DOL in the void peak remained fairly constant at about 50%. In this experiment, the input ratio of PBGT to DOL varied from 0.13:120 to 10.0:120, with the resulting lipid vesicles which varied from less than 1.0 PBGT/vesicle to greater than 400 PBGT/vesicle on the average. The vesicles appeared unilamellar and have an average diameter of $1000\text{\AA}$ as judged by negative stain electron microscopy. This size was in good agreement with lipid vesicle preparations using a similar method for vesicle formation (80).

When the dialysed vesicles were layered onto a shallow sucrose gradient (0-20%), the lipid and protein radiolabels banded near the top of the gradient, which was characteristic of unilamellar lipid vesicles of this size. Less than 2% of the protein could be detected farther into the gradient or pelleted at the bottom (data not shown). Both the electron microscopy data and the sucrose gradient analysis suggest that large stable unilamellar lipid-protein vesicles were formed independent of the protein concentrations used.

Binding of PBGT-Vesicles with Microsac Membranes or RBC Ghosts

The relationship between the PBGT density in the vesicle membrane and the efficiency of vesicle binding to acetylcholine receptor enriched microsac membranes was a key question. This experiment focused on the relevance of this model system and the multivalent binding

hypothesis. The ratio of PBGT to vesicle was varied from 10 to 700. RBC ghosts were used as a control membrane. In these experiments, the amount of liposomes added to the membrane vesicles was kept constant. It was evident that at high concentrations of PBGT greater than 300 PBGT per vesicle, there was a marked increase in the total amount of both radioisotope labels (protein and lipid) associated with the microsacs (Figure 13). At concentrations less than 300 PBGT per vesicle, no significant binding was seen. Such "threshold" phenomena was not observed with the RBC ghost membranes, although some non-specific binding was evident with liposomes of high PBGT/vesicle. In Figure 14, the same data were expressed as the percent of total added PBGT and lipid that become bound to the microsacs or RBC ghosts. At all concentrations, the percentage of ^{125}I -PBGT associated with the microsac fraction was always greater than the percentage of ^3H -lipid. However, at the highest ratio of PBGT per vesicle, greater than 20% of both isotopes associated with microsacs whereas only 7% of either marker associated with the control RBC ghost membranes.

Discussion

Fatty Acid Conjugation of α -bungarotoxin and its Purification

The coupling reaction between ^3H -NHSP and ^{125}I - α BGT was shown in Figure 8 (see page 51). The ^3H -NHSP concentration was varied over a wide range while the toxin concentration remained fixed. Optimum conditions to produce a mono-conjugated product (when the palmitic acid to protein mole ratio of the product was one) occurred when the initial molar ratio of ^3H -NHSP to ^{125}I - α BGT was 3:1. Under these

Figure 13. Binding of PBGT-Vesicles to Microsac or Red Blood Cell Ghost Membranes.

Vesicles containing various amounts of PBGT were used. Binding of both ^{125}I -PBG and ^3H -lipid are indicated.

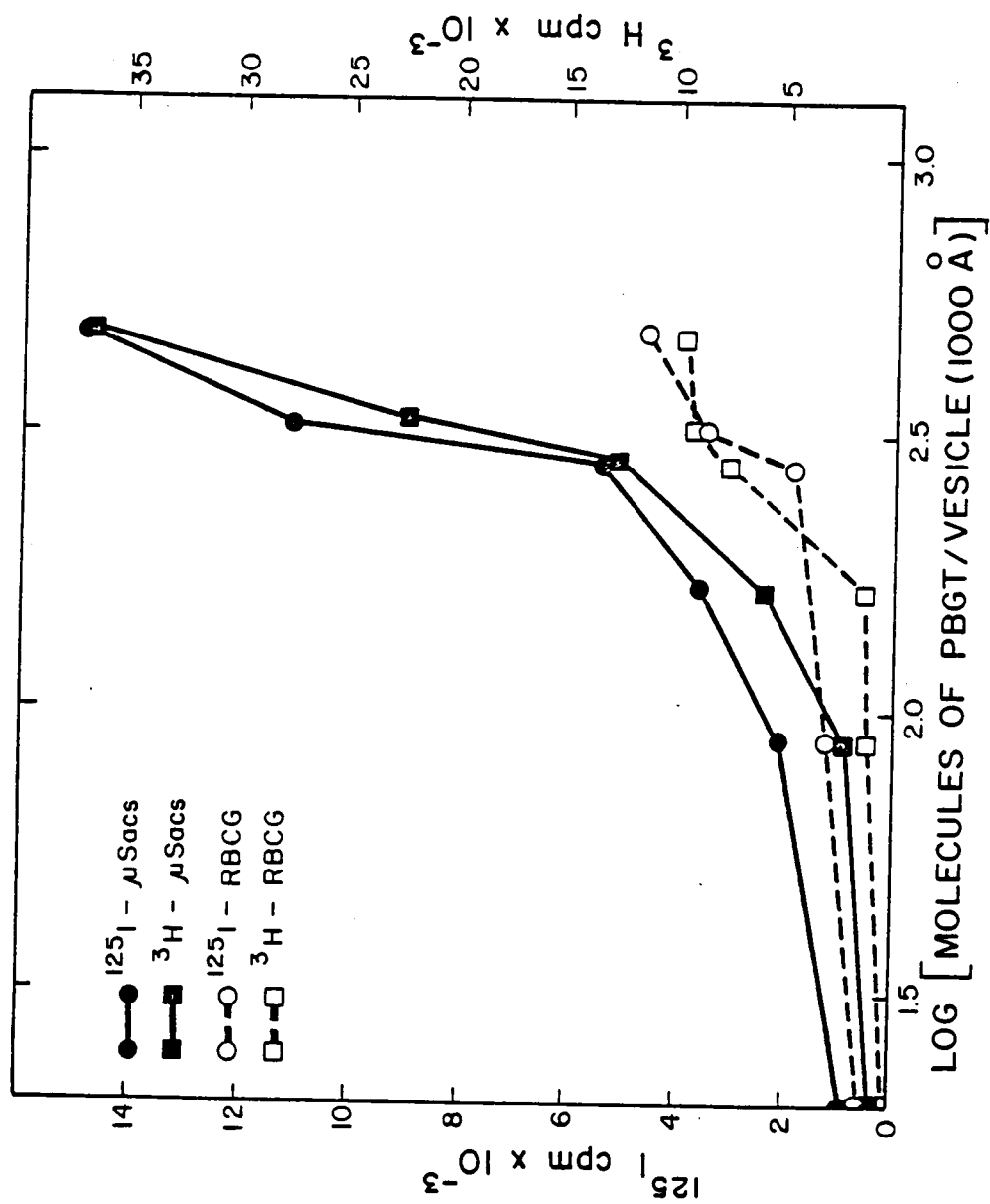


Figure 13

Figure 14. Binding of PBGT-Vesicles to Microsac or Red Blood Cell Ghost Membranes over the Entire Concentration Range.

The data in Figure 13 are expressed as percentages of total radioactivity added.

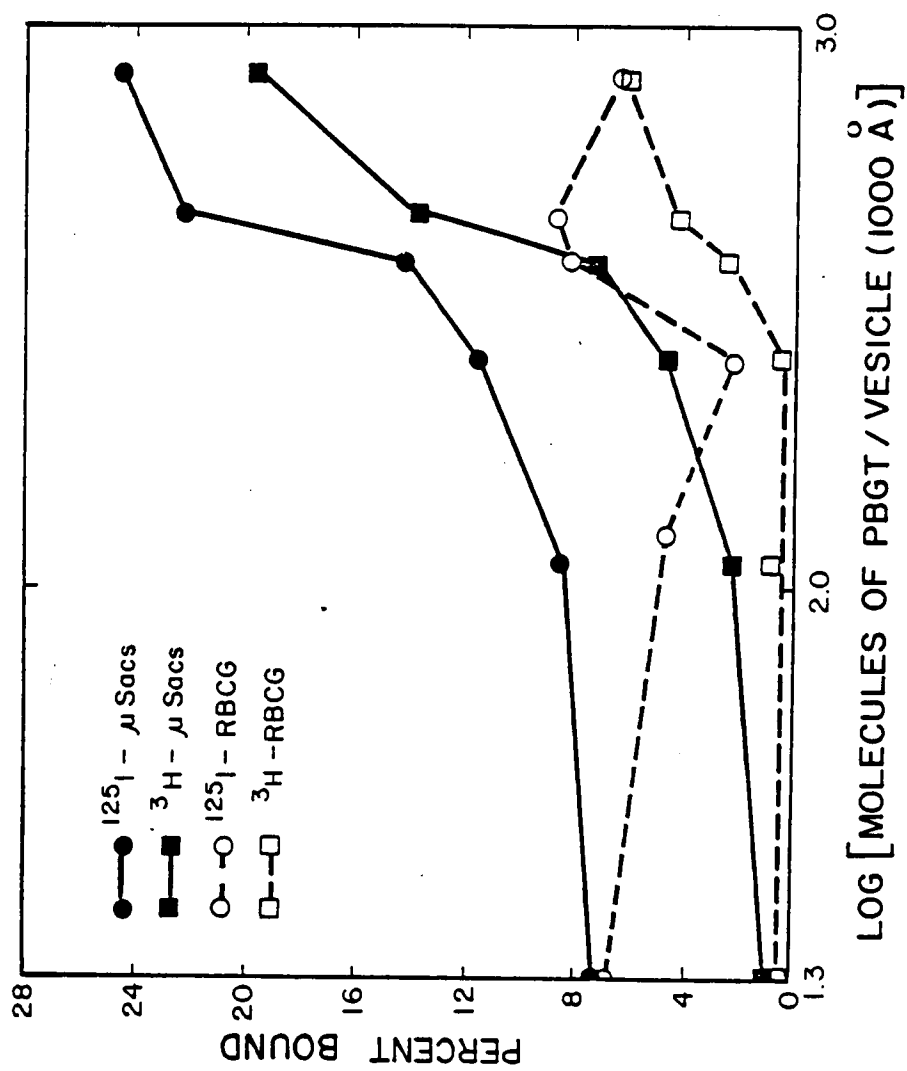


Figure 14

conditions, a purified product of the reaction has been identified as mono-palmitoyl- α -bungarotoxin, PBGT. The yield of PBGT (>60% of the initial protein added) was very high. This was in contrast to the relatively poor yields reported for the anhydride reactions (about 15%) as discussed in Chapter II (page 11). The ^3H -NHSP coupling reaction was much better controlled when compared to the anhydride reaction. The hydrolytic degradation of ^3H -NHSP was monitored spectrophotometrically in the absence of protein (81-82). The half-life for this reagent was about four hours. This slow hydrolysis was in contrast to the rapid hydrolysis of all fatty acid anhydrides used in the first coupling reaction described in Chapter II (page 11). Typically, these reactions go to complete hydrolysis in a few minutes. It has been demonstrated that activated esters of N-hydroxysuccinimide preferentially attack primary amine groups (83-84). It was most likely that the coupling of palmitic acid via an amidization reaction to the epsilon amine group of one of the six available lysines occurred. It was possible that not all of these lysines would be available for coupling to palmitic acid. However, using high concentrations of the coupling reagent compared to toxin, a protein product has been isolated which has undergone multiple conjugations with palmitic acid.

The evidence for the homogeneity of the purified PBGT was based on four criteria. Firstly, it appeared as a single protein band on SDS gel. Secondly, the ratio of palmitic acid to toxin was approximately one. Thirdly, it associated with liposomes when chromatographed on Sepharose 4B (Figure 11, page 59). The small amount (less than 15%) of the unassociated protein probably represents the underivatized

toxin. In this sense, the association between PBGT and liposome acts as a final step of purification. Finally, the PBGT binding to the TSTR demonstrated a single class of ligand binding as one single straight line was obtained in the Scatchard plot (Figure 10, page 56). We therefore conclude that the purified PBGT was largely, if not entirely, homogeneous, the upper limit of contamination by the underivatized α BGT was probably 15%.

Binding Affinity of PBGT to Triton Soluble Torpedo Receptor (TSTR)

It was clearly evident that the affinity of PBGT for TSTR was altered compared to native toxin. This altered affinity of PBGT for the receptor was the result of the conjugation reaction. However, there was only about one order of magnitude difference between the affinities of PBGT and ^{125}I - α BGT for TSTR. Since this affinity ($K_D \leq 10^{-7}$ M) was relatively high compared to most biological binding systems, sufficient binding affinity remains such that PBGT will be useful as a membrane-bound targeting ligand. More importantly, these two ligands appear to be binding to the same class of receptors. This was illustrated in the Scatchard plots shown in Figure 11 (page 59). Both ligands displayed linear plots which intersected the axis at the same point. This intercept represented the total number of receptors available for binding. When the affinity of PBGT for the receptor was compared to the affinity of ^{125}I - α BGT shown in Figure 11 (page 59) and α BGT in Figure 10 (page 56), there was close agreement in the magnitude of difference between PBGT and either native toxin or the iodinated species. This difference, which was about one order of magnitude, was most likely attributed to a slight conformational change within the protein molecule.

Importance of PBGT Surface Density on the Efficiency of Site-Specific Vesicle Binding

The most important questions relative to developing a model drug delivery system were those questions which define and characterize the system for optimum efficiency. The following question was the most fundamental and important one in that respect: What quantity of PBGT must be incorporated into the vesicle bilayer before these vesicles become efficient in site-specific binding? This was a particularly interesting question. The underlying assumption was that there exists some lower limit below which a targeting vesicle was no longer an efficient binding unit. The model system developed in this report was designed to make a direct evaluation of this assumption. This threshold would help define the architectural parameters important to the efficient design of a site-specific targeting vesicle. Using this system, one can control the composition of the PBGT vesicle and then evaluate its efficiency to bind with the microsac membranes. An example of this experiment is seen in Figures 13 and 14. In Figure 13, there exists a lower limit below which targeting was no longer efficient. In this experiment, using PBGT vesicles of average size, $1000\text{\AA}$, the lower density limit was approximately 300 PBGT molecules per vesicle. At this limit, there was one PBGT per every 140 molecules of lipid. This limit represented a surface density of 1.59×10^{-12} moles/cm² or 3.0×10^7 molecules/cm². Since the method we used to incorporate PBGT into liposomes probably yielded 50% of PBGT facing the inner surfaces of liposomes, this number should be reduced to half, or 1.5×10^7 molecules/cm².

There was a marked increase in the binding efficiency of PBGT-vesicles to microsacs when high ratios of PBGT to lipid exist in the vesicle bilayer. The fidelity of this site-specific binding of the PBGT vesicles for an acetylcholine receptor enriched membrane was dramatic. The high efficiency of vesicle binding shown for microsacs was in sharp contrast to the small non-specific binding demonstrated in the control, RBC ghost membrane. There was a constant percentage of ^{125}I -radiolabeled bound to the RBC ghost membrane at most concentrations. This was probably an ionic interaction between the positively charged ligand PBGT, and a negatively charged membrane system, RBC ghost.

The concept of targeting implied that the extent of association of both the lipid and PBGT for the target membrane (microsacs) should be the same. However, it was apparent in Figure 14 that the binding of the PBGT was always greater than the lipid. Two plausible explanations for this phenomenon exist. First, not all PBGT were truly incorporated into the vesicle bilayer. The unincorporated toxin molecules bind with the acetylcholine receptors independent of the lipid vesicles, thus elevating the percent of toxin binding over that of the lipids. Secondly, the binding of the PBGT-vesicles to the microsacs was relatively weak such that a portion of the vesicles dissociated from microsacs, leaving the PBGT bound to the receptors. In other words, the PBGT was unrooted from the vesicles. A third possibility was that the distribution of PBGT among the vesicles was not uniform. Vesicles containing more PBGT bound firmly, but vesicles with

few or no PBGT bound loosely or did not bind at all. The result was a higher percentage of PBGT binding than lipids.

The first possibility was unlikely because no significant amount of free PBGT was found when PBGT-vesicles were centrifuged in a 0 - 20% sucrose density gradient. When free PBGT without lipids was centrifuged under identical conditions, most of the toxin molecules aggregated and pelleted at the bottom of the tube. The second possibility was also unlikely because the fatty acyl chain of PBGT should be sufficiently long to stably anchor the molecule into the vesicle bilayer. Although the binding affinity of PBGT to liposomes was not quantitated, it was significantly higher than that of the octanoyl- α BGT ($K_D = 10^{-7}$ M, Chapter 2, page 41), and should also exceed the binding of PBGT to the acetylcholine receptors ($K_D = 2 \times 10^{-7}$ M, this chapter). If any stress force was sufficiently large to break the vesicle-microsac complex, the bond broken should be the one between the toxin and the receptor and not between the toxin and the vesicle, i.e. the toxin would not be unrooted from the lipid bilayer.

We were in favor of the last possibility. First of all, a heterogeneous distribution of fatty acid derivatized immunoglobulin in lipid vesicles prepared with a similar method as the one described here has been observed (85). Furthermore, as shown in Figure 14, the difference in binding between the PBGT and the lipid decreased as the vesicles with a higher average ratio of PBGT were used. In these vesicles, a larger proportion of the population would have the number of PBGT molecules per vesicle that exceeded the lower threshold limit for

microsac binding. This resulted in a reduced discrepancy in the percent of binding of the toxin and that of the lipids. In preparations with lower average ratio of PBGT to lipids, most of the vesicles probably contained little or no PBGT. Only a minor amount of vesicles with the above-threshold number of PBGT per vesicle would bind. In this case, the discrepancy in binding would be large.

The threshold phenomena in liposome binding to microsacs was predicted from the multivalent binding theory discussed in Chapter I. In other words, a positive cooperativity in binding between two membranes (liposomes and microsacs in this case) existed as one examined the extent of binding as a function of ligand or receptor densities in the membrane surfaces. The same theory also predicted that, under the conditions of multivalent binding, the binding was so great that it was essentially irreversible (86-87). One would expect that, based on a mathematical argument, such tight binding cannot be inhibited by soluble ligands. This was indeed the case. In all experiments when vesicles with a high molar ratio of PBGT to lipid were used, only a marginal inhibition of liposome-microsac binding can be demonstrated. However, when the same experiment was carried out with vesicles having ratios of PBGT to lipid well below the threshold value, reasonable inhibition ($\geq 70\%$) could be seen. These results again reinforced the importance of the multivalent binding in the site-specific vesicle binding to membranes or cells.

In summary, palmitic acid derivatized α BGT appeared to be an excellent model for studies on liposome targeting. The synthesis of PBGT was relatively simple and the purified PBGT could be obtained with

high yield. The incorporation of PBGT into lipid vesicles was also straight forward and the resulting vesicles were stable. The ability of this system to demonstrate a multivalent binding to the target membranes has proved its use as a model not only for liposome targeting, but also for studies of membrane-membrane interactions mediated by specific ligand-receptor binding.

CHAPTER IV

CONCLUSIONS

In the final chapter, I will re-evaluate the results of this project. Three main areas will be examined with regard to their contribution toward the field of liposome targeting. Firstly, I will compare the two coupling reactions and their products. Secondly, I will discuss the significance of the present results in relation to the multivalent binding hypothesis. The targeting efficiency of the system will be analyzed. Finally, a summary of the achievements made in this project which further the understanding of the field of liposome targeting will be discussed. Possible future work with this system is also suggested.

Comparison Between the Two Fatty Acid Derivatives of α BGT

In reviewing the results from Chapters Two and Three, it is evident that given a stable protein which has enough secondary and tertiary structures to withstand limited chemical modification, conversion from a highly hydrophilic protein into a lipophilic membrane protein is a simple procedure requiring only the selection of a good coupling reaction. Using octanoic acid anhydride, α BGT can be converted into OBGT. Using NHSP, α BGT can be converted into PBGT. These two proteins are easily purified. They also have several common characteristics. Both proteins retain their acetylcholine receptor binding affinity. Only a nominal decrease in this affinity is shown for either protein when compared to native α -bungarotoxin. There

is little difference in the K_D of these two protein conjugates. Importantly, these conjugated toxins appear to be binding to the same class of receptors as native toxin. Although both OBGT and PBGT demonstrate a large affinity for the vesicle lipid bilayer, one would expect that PBGT binds to liposomes more firmly than OBGT due to a difference in the fatty acyl chain length. Concentration levels within the lipid bilayer for OBGT and PBGT can be raised to very high levels.

Although these two proteins have shown very similar behavior both chemically and physically, they are different in two ways. The yield of OBGT is much lower when compared to PBGT. Secondly, the stability of the OBGT vesicle is not as good as the PBGT vesicles. OBGT vesicles will aggregate upon storage at high concentrations of the protein in bilayer. PBGT tends to be more hydrophobic when compared to OBGT.

Significance of the Present Work on Relation to the Multivalent Binding Hypothesis and to Liposome Targeting

Our model system has clearly shown the enhancement of the site-specific binding of liposomes to a model target cell. Perhaps the most important finding of this work is the demonstration of the involvement of multivalent binding. Only under conditions which favor the formation of multiple bonds between the liposomes and the target membranes, can a significant target specificity be achieved. This observation has defined the necessary conditions for successful liposome targeting. Independent of the targeting molecules chosen, these conditions have to be satisfied.

A threshold value in the number of targeting molecules per liposome for significant liposome binding is predicted by the multivalent binding hypothesis (88-89). The same theory also predicts that the overall binding constant is so high in multivalent binding that the soluble ligand is difficult to compete. This notion can be understood from a simple calculation pertinent to the present model system.

The concentration of membrane bound reactants will be very high in the space near the membrane. In a local region near the membrane, the number of molecules per unit volume will be the number per unit area on the membrane divided by the thickness of the region (10-100Å) next to the membrane within which the molecules are confined (90). The surface density of the acetylcholine receptor in receptor-rich membranes is 12,000 to 15,000 per μm^2 (91-92). If the binding sites for the receptor are located within a 20Å band of the surface, the local concentration of receptors is calculated from the following:

Given:

Receptor surface density 13,500/ μm^2 (average) located in 20Å band around surface.

Then:

$$13,500/\mu\text{m}^2 = 1.3 \times 10^{12} \text{ receptors/cm}^2$$

Receptor concentration =

$$\frac{1.3 \times 10^{12} \text{ receptors/cm}^2}{2.0 \times 10^{-7} \text{ cm}} = 2.6 \times 10^{19} \text{ receptors/cm}^3$$

$$= 43 \text{ mM}$$

A similar calculation for the liposome-bound PBGT for those showing multivalent binding (typically 500 PBGT molecules per liposome of 1000Å in diameter) gives a local concentration of PBGT 6.6 mM. In

view of the low dissociation constant for the binding of PBGT to the detergent solubilized receptor ($K_D = 2 \times 10^{-7}$ M), practically all PBGT will be bound to the receptors once the liposomes come to a close proximity of the microsacs. Furthermore, it also predicts that the free soluble toxin can only be competitive at a concentration in the mM range, an economically prohibitive amount. However, if the number of PBGT per vesicle drops significantly below the threshold value, lower amounts of free toxin should be inhibitive. This is indeed the case. The binding of the multivalent PBGT-vesicles to microsacs cannot be significantly inhibited with the free toxin up to about 100 μ M in concentration. On the other hand, about 70% inhibition can be achieved in experiments using vesicles of low PBGT concentration, less than 20 PBGT's per vesicle.

The low value of K_D measured for the binding of PBGT to receptors in detergent is probably an overestimate in the strength of binding when PBGT and receptors are located in membranes. First of all, from a thermodynamic point of view, the loss of one degree of freedom when going from a three-dimensional solution to a two-dimensional membrane will definitely decrease the binding capacity of a molecule. This loss of freedom in motion may be associated with the rotational restrictions placed upon the molecule when locked onto the lipid bilayer. Secondly, certain steric restrictions and strains may be placed upon protein ligands incorporated into the lipid bilayer. Finally, geometrical features of the microsac and the vesicle may play important roles in further decreasing the binding efficiency. It is likely, however, these losses of binding affinity can be compensated and exceeded when a multivalent binding situation is established.

Future Research Directions

Obviously, the actual use of liposome as a site-specific drug delivery system is far beyond the scope and the purpose of this work. The present work explores only the fundamental physico-chemical bases underlying the ambitious attempt of liposome targeting. It is the first step of a long journey. However, some results of this work have already strongly influenced other attempts of liposome targeting. For example, the conjugation of immunoglobulin with long chain fatty acids using a method similar to the one used for the preparation of PBGT has successfully been accomplished in this laboratory (93). Some lectin proteins, such as the wheat germ agglutinin, can also be coupled with the fatty acids (94). It is likely that the methodology developed in this work will have a long reaching influence in many areas of membrane research including liposome targeting.

Within the framework of the present model system, a number of future research directions are apparent. Firstly, a rapid kinetic assay for liposome-microsac association is needed in order to explore the kinetic aspects of the binding. Secondly, the influence of the mobility of the ligand and of the receptor on the binding of liposome to microsacs deserves careful studies. Both the rotational and the lateral mobilities of the proteins may play important roles for the establishment of the multivalent binding. Thirdly, the dependence of the overall binding on the intrinsic binding affinity of the individual ligand-receptor pair should be explored. The snake toxins of lower binding affinities, e.g., α -naja toxin, may be useful in this regard. Fourthly, reconstituted liposomes containing purified acetylcholine

receptors may be used to replace the microsacs in the binding assays. The surface density, rotational and lateral mobilities of the receptor can be better controlled in the reconstituted system than the native microsacs. Finally, cells containing high levels of surface acetylcholine receptors, e.g. the myotubes, should facilitate the studies on the metabolic fate of the liposomes once becoming bound to cells. It is important to know if the trapped contents of the bound liposomes can be transferred to cells by mechanisms such as fusion and endocytosis.

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