The isolation and characterization of antigenic glycopeptides from electroplax membranes of Torpedo marmorata.

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THE ISOLATION AND CHARACTERIZATION OF ANTIGENIC GLYCOPEPTIDES FROM ELECTROPLAX MEMBRANES OF TORPEDO MARMORATA

Submitted by

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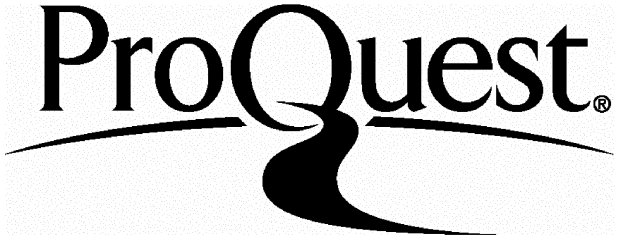

DEDICATION

This thesis is dedicated to my wife Alison, and my children, David and Susannah.

LIST OF ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
AChR	acetylcholine receptor
nAChR	nicotinic acetylcholine receptor
Ab	antibody
Ag	antigen
ATPase	cation exchange mechanism enzyme
αbgt	alpha bungarotoxin
BSA	bovine serum albumin
Ci	curie
nCi	nano-curie
Con A	concanavalin A
cyt c	cytochrome c
DEAE	diethylaminoethyl
EAMG	experimental autoimmune myasthenia gravis
EDTA	ethylene diamine tetracetic acid
glc	gas-liquid chromatography
^{125}I	iodine 125
IE	immunoelectrophoresis
IgG	immunoglobulin G
M	mobility
MBTA	4(N-maleimido)benzyl trimethylammonium iodide
MPTA	4(N-maleimido)phenyl trimethylammonium iodide
Mr	relative molecular mass
MW	molecular weight
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAGGE	polyacrylamide gradient gel electrophoresis
PAS	periodic acid-schiff
PMSF	phenyl methyl sulphonyl fluoride
RIA	radioimmunoassay
SDS	sodium dodecyl sulphate
TA	total activity
TEMED	tetra ethyl methyl ethylene diamine
tlc	thin layer chromatography
TMS	trimethyl silyl ether
YADH	yeast alcohol dehydrogenase

CONTENTS

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I acknowledge the active encouragement, help and advice I have received from my supervisors Drs. R. Harrison and G.G. Lunt. I am also indebted to Dr. T. Barkas and Mrs. C.M.J. Watson for their help with ^{125}I-labelled toxin binding studies and radio-immunoassays. I would like to thank too, those of my colleagues, especially Drs. G.H. Farrar and T. Barkas who provided many helpful and stimulating discussions.

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ABSTRACT

Membrane fragments were prepared from electric organs of the electric ray, Torpedo maromorata and shown to contain exposed acetylcholine receptor (AChR) as evidenced by their binding of radiolabelled α-bungarotoxin and Naja naja toxin. When the membranes were labelled with the affinity ligand ^{3}H -MBTA, specific for the acetylcholine binding site of AChR, and subjected to SDS-PAGE analysis, 12 major protein components were detected, only one of which (M.W. 40,000) contained the radiolabel.

The protein components of the purified membranes were examined by using a number of approaches.

Membranes solubilized in Triton X-100 showed a single precipitin line when analyzed by double diffusion using rabbit anti-(Torpedo membrane) antisera and the presence of four to six antigenic components when analyzed by rocket and crossed immunoelectrophoresis using the same antisera.

Treatment of purified Torpedo membrane fragments with pronase released soluble glycopeptides which in double diffusion experiments against rabbit anti-(Torpedo membrane) antisera, gave two precipitin lines, one of which showed a reaction of identity with the single line given by solubilized whole membrane fragments. The soluble glycopeptides were fractionated on Sephadex G-50 when three hexose-containing protein peaks A, B and C were separated. Peak A, showed antigenic cross reactivity with rabbit anti- (Torpedo membrane) antiserum in immunodiffusion and electrophoresis assays and inhibited the precipitation o· ^{125}I-labelled

purified Torpedo acetylcholine receptor by rabbit anti-AChR IgG.

Glycopeptide peaks A, B and C when analysed by gas chromatographic and colourimetric techniques all showed the presence of fucose, mannose and galactose, but not sialic acid. Peaks A and B contained glucose while peak C showed additionally glucosamine and galactosamine. These sugar compositions were compared with that obtained by similar analysis of affinity purified AChR from Torpedo marmorata.

PREFACE

The successful isolation and characterization of the nicotinic acetylcholine receptor (AChR) has been facilitated by the availability of a unique tissue - the electric organ of several species of electric fish. The electroplax cells of electric tissue from Torpedo marmorata are exclusively cholinergic in their innervation and comprise densely packed arrays of receptor molecules providing a rich source of materials.

The human disease myasthenia gravis and its animal model, experimental autoimmune myasthenia gravis both involve an autoimmune response to antigenic determinants associated with the AChR at the subsynaptic membrane of the neuromuscular junction. It is accordingly of interest to learn something of the structural basis of the antigenicity exhibited by membrane fragments rich in receptor material.

This thesis details the methods employed to isolate and characterize those macromolecular components of electroplax membrane fragments which may be obtained by proteolysis and detergent solubilization. It is not intended to be a definitive work in any sense, but represents a generalized rather than a specific approach to the problem.

The pace of research in this area has been such that the conclusions drawn here may appear to be superficial, but it must be remembered that the experimental work was completed in 1978 and much has been added to our knowledge of this particular

system during the last three years. Advances in preparative techniques have led to fractionation methods which provide membrane fragments extremely rich in receptor material, and the development of immobilized ligand/toxin purification techniques have raised to a very high level the degree of receptor preparation purity now being achieved.

The isolation of macromolecular components from these highly purified membrane fragments has been developed by a number of research groups working in this field, with contributions from the laboratories of Raftery, Karlin and Changeux being particularly notable. There is general agreement concerning the molecular weight of the component which is covalently labelled by reagents specific for the receptor site, but the MW values for the β, γ & δ polypeptide chains vary considerably. There has recently been the introduction by Wennogle and Changeux of a value of 43,000 for the β chain which compares with the 48,000 quoted by other sources. A model published by Wennogle and Changeux depicts this subunit as being devoid of carbohydrate and buried in the membrane interior presumably accessible only following membrane disruption.

It is well estabished that the AChR is a glycoprotein but some of the recent work from the biochemistry group at Bath, has indicated that the contribution made to antigenic activity by carbohydrate groups is perhaps not as significant as was previously believed.

Some of the work detailed in this thesis has been published in Biochem. Soc. Trans. Vol. 6, p. 639.

INTRODUCTION

1. ELECTRIC FISH AND ELECTRIC ORGANS

The successful isolation and characterization of the nicotinic acetylcholine receptor (nAChR) has only been made possible by the availability of an unusual tissue: the electric organ of several species of so-called "electric fish". Because of its completely cholinergic innervation, electric tissue from the marine ray Torpedo has proved a rich source for the purification of the nAChR (typically 40 mg. protein per kg. organ; Valderama, et al., 1976).

The ability of Torpedo to administer numbing shocks has been known since Roman times, and was even the subject of a poem "De Torpedine" by Claudian (370-408 AD). A primitive form of electro-convulsive shock therapy involved placing a live Torpedo on the patient's head.

Bioelectric potentials were first described around 1757 by Michel Adanson, who compared the sensation felt on touching the Malapterus, to a discharge from a Leyden flask. Some twenty years later John Walsh demonstrated that the shock from a Torpedo was an electrical discharge, and at about the same time Williamson made corresponding observations on the freshwater eel Electrophorus. It was only after Galvani's revelations concerning nerve-muscle preparations however, that biologists became really interested in studying electric fish. During the last two years of his life, Galvani himself worked with Torpedo.

Throughout the last century, a number of physiologists analysed the many different aspects of both the electrical discharge and the unique structural properties of the electric organs of the fish. The most important fact from a physiological point of view was that

bioelectric potentials were generated in the same way as in nerve and muscle. This was recognised, and particularly stressed by Du Bois-Reymond in 1877. His conviction was that an analysis of the phenomenon of electrical discharge in such fish would eventually lead to a better understanding of the electrical manifestations of nerve and muscle.

Biochemical studies on the nAChR have been performed primarily with electric organs from two fishes: the fresh water teleost Electrophorus electricus and the marine elasmobranch Torpedo marmorata. An electric organ is essentially composed of compartments, each containing an electric plate - the electroplaque - these being arranged in columns. The potential which can be developed by a single electroplaque is about 140 mV, around the same order of magnitude as that found in ordinary nerve and muscle fibres. A high voltage is only obtained by a series summation of the tiny voltages produced by each electroplaque cell, operating in a similar fashion to the voltaic pile. Volta himself recognised the analogy. When his paper was read before the Royal Society in 1800, describing his pile he wrote that he wished to call it an "artificial electric organ".

Several species of fish possess electric organs and the variations in the discharge from each species are a function of the shape and size of the organs themselves. The species with the most powerful electric organ known - Electrophorus electricus - has 5,000 - 6,000 electroplax arranged in series from the cephalic to the caudal end of the organ. The organs occupy about half of the caudal region of the body and weigh just less than one-half of the total body weight (e.g. approximately 500 g. for a specimen 1 m. long).

Anatomists distinguish three organs in Electrophorus, the electroplax cells deriving from skeletal muscle, receiving their innervation from spinal electromotoneurons with a segmental distribution. The electric organs in Torpedo constitute two large, flat, kidney-shaped masses on both sides of the anterior part of the body, their weight ranging from one sixth to one quarter of that of the fish. Embryological studies have shown that the organ derives from striated muscle (specifically modified branchial muscles) and receives approximately 6,000 nerve fibres from an equal number of neurons clustered in lobes on the dorsal part of the mesencephalon (Figure 1).

The electroplaque cell in both species is a giant syncytium containing several thousand nuclei and is highly asymmetrical, receiving innervation on only one face: the ventral in Torpedo and the caudal in Electrophorus. The cytoplasmic membranes of both faces show an increase of surface area as a direct result of the number of invaginations (Luft, 1956; Bourgeois, 1974). In Torpedo the innervation is much more dense than in Electrophorus and the innervated membrane less convoluted so that the subsynaptic areas occupy up to one half of the total surface area. Torpedo electric organs are therefore expected to be much richer in subsynaptic membranes and consequently, in receptor protein than Electrophorus.

The large literature concerning anatomical data and electrical characteristics of these fish has been detailed in reviews and articles by Rosenberg (1928); Fessard (1958); Albe-Fessard (1959), Bennett (1961; 1970); Grundfest (1967) and Changeux & Podleski (1968).

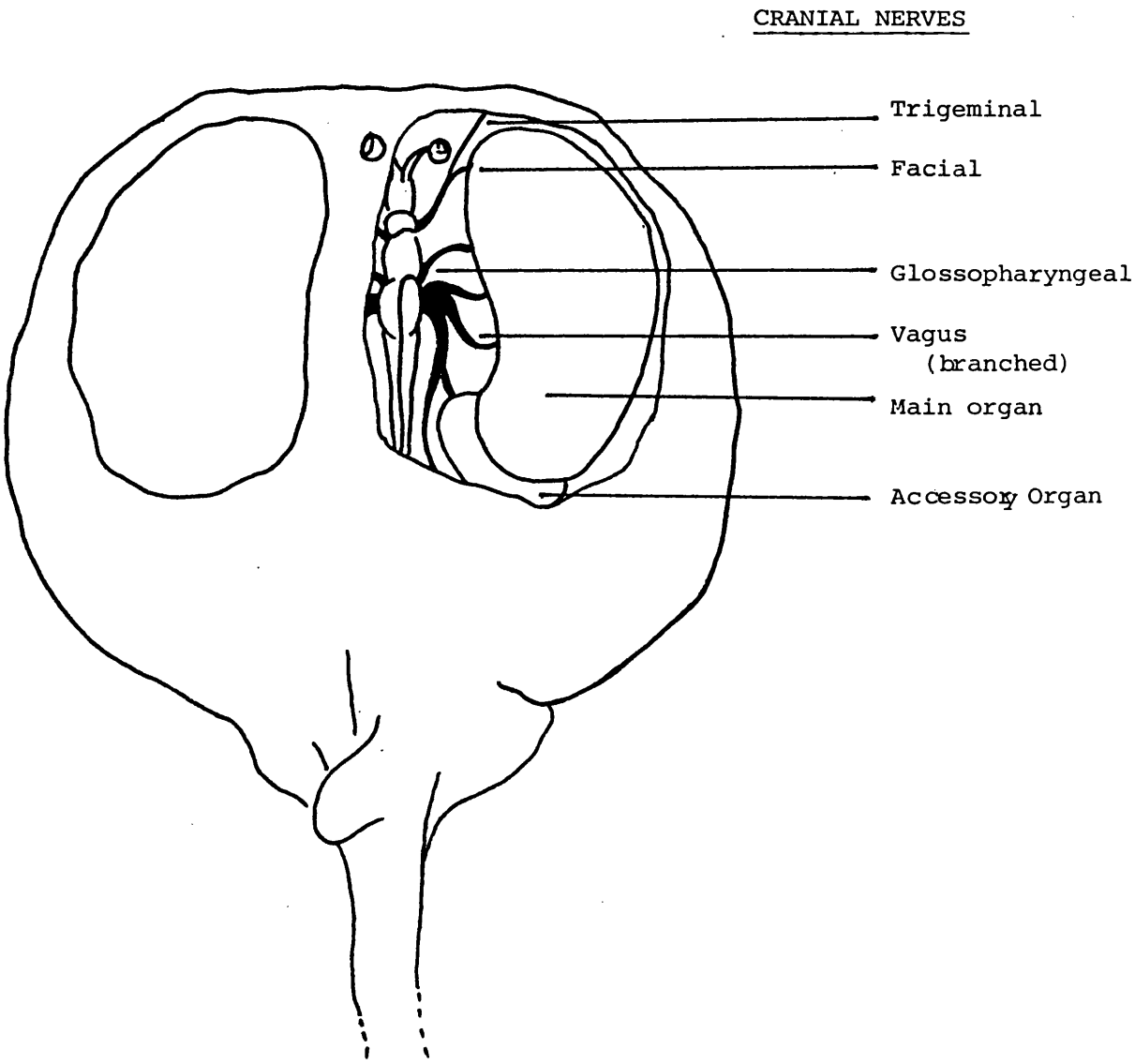


Figure 1. The position of the cranial nerves which innervate the electric organs of Torpedo marmorata.

2. BIOCHEMICAL STUDIES ON THE ELECTRIC ORGAN

Although Meyer in 1937 had proposed that a configurational change of proteins with a rearrangement of acidic and basic groups was the factor responsible for permeability changes to ions during conduction, it was Nachmansohn in 1952 who first postulated that the receptor for acetylcholine ought to be a protein. At this time he had no experimental evidence for such an assumption,which was based entirely on the premise that only a protein would be able to recognise a small ligand like acetylcholine with such specificity. This premise was derived from experimental observations, but those experiments performed in the late 1930's using isolated electroplaque preparations afforded entirely inappropriate conditions for studying the chemical nature of the receptor. The development of monocellular electroplaque preparations by Schoffeniels (1957; 1959) and Schoffeniels & Nachmansohn (1957) however, really paved the way for the study of the nature and properties of the acetylcholine receptor.

During the mid-1950's , Chagas and his colleagues investigated the binding of ^{14}C-labelled gallamine to electric tissue from *Electrophorus* (Chagas *et al.*, 1956; 1958; Chagas, 1959; see also Hasson-Voloch, 1968). Gallamine paralyzes the electrical discharge, and after injecting the eel with the labelled compound and perfusing with Ringer's solution, the organ was homogenized in distilled water. The solution thus obtained retained binding activity after dialysis against water, but lost it if dialyzed against 0.18 M NaCl solution. It was later shown by Chagas (1959; 1962) that the binding was completely non-specific, involving an acidic mucopolysaccharide unrelated to receptor material.

Although Nachmansohn was the first to suggest that the acetylcholine receptor was a protein, it was Ehrenpreis, (1959; 1960) who lead with a claim for the isolation of a receptor protein from eel electric organ. In 1962 however, he abandoned this claim and Beychok in 1965 showed that the "homogeneous fraction" prepared by Ehrenpreis could in fact be electrophoretically sub-fractionated into several components, none of which could be distinguished either by protein composition or curare binding.

For reviews on these early studies see Hasson-Voloch (1968) and De Robertis (1971).

Nachmansohn's analogy between the binding specificity of his hypothetical receptor and the active sites of enzymes, was further extended in 1963 with the publication by Monod et al.of their observations concerning allosteric proteins and cellular control systems (see also Monod et al., 1965). Changeux and his colleagues were the first to recognise certain common properties of regulatory enzyme systems and excitable membranes. The biological activities of both depend on the threshold concentration of the regulatory ligand, and both exhibit cooperative phenomena (Changeux et al., 1967). The dose response curve of the elctroplax membrane to receptor activation was demonstrated by Higman et al.(1963) to be sigmoidal in shape. Changeux interpreted this in terms of allosteric systems and cooperativity (see also Changeux & Thiery, 1968) while Karlin (1967) discussed ideas along similar lines.

In 1968, Changeux & Podleski carried out detailed analyses of the responses to acetylcholine and its congeners by the electroplaque membrane. Dose response curves obtained for different activators all

had the characteristic sigmoidal shape, with a Hill coefficient of 1.7 - a widely accepted indication of the cooperative nature of the response.

2.1 Acetylcholine Receptor - Affinity Labelling and Binding Studies

In much of the early work concerned with the biochemical isolation of the nicotinic acetylcholine receptor (nAChR), some use was made of substances that bind to the receptor, usually in a specific manner. Chagas and Nachmansohn used curarifom ligands while Takagi and also Turpaev both used relatively non-specific agents in their investigations (Turpaev & Nistratova, 1959; Turpaev, et al., 1964; Takagi et al., 1965; Takagi and Takahashi, 1964.

Time affinity labelling of the receptor was first attempted in 1968 by Changeux et al., using p-trimethylammonium-benzenediazonium fluoroborate (TDF), a structural analogue of phenyltrimethylammonium and a potent AChR activator. The reasoning was that such a compound would form a reversible complex with the receptor's anionic site, the diazonium group forming a covalent bond with a residue near the active site. This exposure to TDF produced an irreversible block of the response to receptor activator. Experiments by Mautner and Bartels in 1970 however, suggested that it is the positively charged diazonium group which is attracted to the receptor subsite. A more potent affinity label - 4(N-maleimido)-phenyltrimethylammonium iodide (MPTA) was used by Karlin and Winnik (1968) on electric tissue following treatment with dithiothreitol (Karlin, 1969).

The development of maleimide derivatives as affinity labels was

based on the discovery that the AChR contains an easily reducible disulphide bond seemingly close to the active site. Reduction of this bond using dithiothreitol markedly altered the pharmacological specificity of the receptor. Reoxidation lead to a full reversal of the effects of reduction (Karlin, 1969; 1974; 1977). The sulphydryl groups formed after reduction are susceptible to alkylation by N-ethylmaleimide which prevents the reversal by oxidizing agents of this reduction. Maleimide derivatives of acetylcholine act in a similar fashion, such quaternary ammonium compounds alkylating receptors at much higher apparent rates than non site-directed agents.

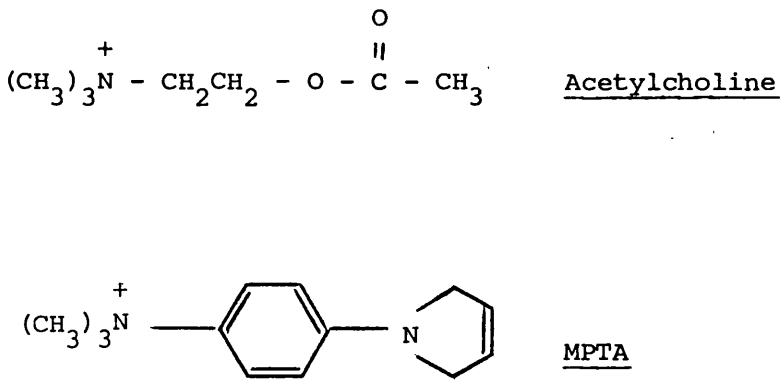


Figure 2. The Chemical Formulae of Acetylcholine and 4-(N-maleimido)-phenyltrimethylammonium

Tritiated 4-(N-maleimido)benzyltrimethylammonium has been used to determine the quantity of AChRs in intact electroplax, and to identify membrane bound detergent,solubilized and purified receptors (Karlin, 1977).

Receptor binding studies were performed by O'Brien and Gilmour in 1969 using homogenates of electric tissue from Torpedo marmorata

which bound tritiated muscarone in a reversible manner (see also O'Brien et al., 1970). This binding was shown to be inhibited by drugs known to combine with nicotinic receptors such as d-tubocurarine, nicotine and acetylcholine itself. Similar studies on homogenates from Electrophorus gave comparable results (Eldefrawi et al., 1971b).

Acetylcholine has not commonly been used as a label for receptors because of its susceptibility to hydrolysis, but Eldefrawi et al. (1971) have shown that a satisfactory measure of binding to electric tissue could be obtained if organophosphorous inhibitors of the esterase were employed.

The biochemical isolation of the nAChR from electric tissue has been greatly facilitated by the use of certain elapid snake venom toxins which occupy the ACh binding site with great affinity and specificity (Chang & Lee, 1963; Lee & Chang, 1966; Lee et al., 1967; Lee, 1972; 1973).

Those toxins which have been most commonly used are: α-bungarotoxin (αbgt) from the krait Bungarus multicinctus, α toxin 3 from the cobra Naja naja siamensis and an α toxin from the spitting cobra Naja nigricollis. These toxins bind to the AChR either at or very near the ACh binding site and act as competitive antagonists in a similar manner to d-tubocurarine, despite their dissimilarity to ACh analogues (Briley & Changeux, 1977; Prives et al., 1972).

Both αbgt and the toxin from Naja nigricollis were employed by Changeux et al. (1970a; 1971) to block the response to both applied carbamylcholine and decamethonium. Miledi et al. (1971) experimenting

with αbgt binding to electroplax membrane fractions gave values of 1100 pmoles per gram protein, while Cohen and his colleagues (1972) using cobra toxin published results of 550 p moles per gram protein (see also Tamiya & Takasaki, 1968). The binding of radioactively labelled snake venom α toxins has been widely accepted as a specific marker for the nAChR, although Karlsson et al. (1972) and Raftery (1973) have shown that more than one type of αbgt binding component exists in the excitable membrane.

2.2 Receptor Solubilization

During the early 1970's, a number of investigators demonstrated that the detergent treatment of electric tissue membrane fragments solubilized a component which retained its binding activity for neurotoxins and other receptor labelling agents (Changeux et al., 1970b; Miledi et al., 1971; Meunier et al., 1971; Raftery et al., 1971; Eldefrawi et al., 1972; Klett et al., 1973). Generally, from the published data, it is clear that extraction with mild detergents such as Triton X-100, Lubrol WX, Tween 80 and deoxycholate, solubilized material with properties similar to the receptor present in the particulate fraction, but the quantitative binding characteristics differed. Discrepancies were also discovered by Meunier and Changeux (1973) during their purification of the nAChR from Electrophorus using affinity chromatography. When compared with material solubilized by deoxycholate (Changeux et al., 1970b; 1971) the purified material had forty times the affinity for decamethonium and ten times that for carbachol and MPTA. It was thus assumed that the environment of the receptor had a profound effect on drug binding (see also Changeux 1975). Of the mild detergents available, Triton X-100 has been

used successfully in the purification of receptor material from many sources. Meunier et al. (1974) purified eel receptor some 300 fold from a Triton solution extract of electroplax membrane fragments, and on the basis of this and many other reports of receptor extraction using mild detergents, the nAChR has been classified as an integral membrane protein

Many researchers have combined the techniques of detergent solubilization and affinity binding of snake neurotoxins to isolate the nAChR from electric tissue using affinity columns, (Karlsson & Heilbronn, 1972; Bisecker, 1973; Klett et al., 1973; Boulter & Patrick, 1977) while others have employed quaternary ammonium compounds as affinity ligands (Schmidt & Raftery, 1973; Meunier et al., 1974).

An alternative approach to receptor solubilization was taken by De Robertis and his colleagues (for review see De Robertis, 1971) who pioneered the use of organic solvents - particularly chloroform/ methanol mixtures - to extract hydrophobic receptor material specific for certain transmitters, and especially the nAChR from Electrophorus and Torpedo electric tissue. (see also De Robertis, 1973; O'Brien et al., 1972; Hall, 1972).

The extraction of a proteolipid from electric tissue that bound both acetylcholine and hexamethonium was described by La Torre et al., (1970). However, the binding studies were performed in organic solvents rather than aqueous solution and the use of non-polar solvent mixtures for experiments with highly polar drugs brought these investigations much criticism. The elution of this proteolipid labelled with a radioactive ligand from a column of lipophilic Sephadex LH20, was

criticised by Levinson and Keynes (1972) on the basis that the cholinergic proteolipid protein peak was artefactual. This was later refuted however by Donellan and Cattell (1975). The fact that large amounts of receptor material were obtained by organic solvent extraction was a surprising feature of this technique, and the group of De Robertis claimed that values obtained from binding studies were actually consistent with data published by other workers. There were however, many inconsistencies when compared to results obtained by Eldefrawi et al. (1971). Fiszer and De Robertis (1972) showed that a solution of the proteolipid in chloroform took up αbgt, d-tubocurarine, hexamethonium and ACh while pretreatment with αbgt did inhibit both decamethonium and ACh binding. Despite this there have been several serious objections to the technique. Potter (1973) found that the toxin-receptor complex was not extracted with chloroform/methanol, although apparently the cholinergic proteolipid after transfer to an aqueous detergent solution could bind the toxin (De Plazas & De Robertis, 1972).

Similar attempts have been made in other laboratories (see Barrantes et al, 1975; Heilbronn, 1975; Karlin, 1973; De Robertis et al., 1976) provides strong support for the proteolipid.

It is interesting to note, however, that Kametari et al. (1975) using electric tissue from the Japanese ray Narke japonica found that most of the chloroform:methanol soluble ACh binding material could be extracted from the fraction rich in acetylcholinesterase and very little from the receptor-rich membrane fractions.

2.3 Purification and Characterization of the nAChR from Electric Tissue

Among the first studies in this area were reported by Miledi (1971), who labelled membrane receptors with iodinated αbgt and dissolved the whole complex in Triton X-100. The use of Sephadex gel filtration and density gradient centrifugation showed that the receptor material had a very high molecular weight, and could be separated from the esterase with ease. Conventional protein purification procedures have given a significant purification of both the free receptor and the toxin-receptor complex, when carried out in neutral detergent solution. These techniques have been particularly successful with the high specific activity (1,000 - 2,000 nmole α toxin binding sites per gram protein) crude extracts from Torpedo electric tissue (Potter, 1973). The low specific activity (20 - 80 nmoles α toxin per gram protein) of crude extracts from Electrophorus tissue made purification by conventional procedures that much more difficult. Because the purification factors required were several hundredfold, many researchers turned to affinity chromatography to improve both the purification factors and the yields.

Two approaches to purification using affinity chromatography were adopted: (i) the conjugation of Naja α toxin to agarose beads as an affinity absorbent from which bound AChR could be eluted using a cholinergic ligand. Selective absorption of receptor protein to the beads occurred while acetylcholinesterase (AChE) remained in solution (Meunier et al., 1971). Unfortunately a quantitative release of the receptor from the column could not be effected until the toxins from Naja naja or Naja naja siamensis, which bound the receptor site

with a lower affinity than Naja nigricollis toxin, were used to prepare the columns (Karlsson et al., 1972; Klett et al., 1973; Eldefrawi & Eldefrawi, 1973; Lindstrom & Patrick, 1974).

(ii) A second type of affinity column was developed concurrently with the snake toxin column (Olsen et al., 1973; Schmidt & Raftery, 1972; Bisecker, 1973; Karlin & Cowburn, 1973) which relied on the conjugation of synthetic cholinergic ligands to agarose beads. The efficient separation of receptor and esterase was achieved by differential elution with either gallamine (Olsen et al., 1972; Meunier et al., 1974),decamethonium (Bisecker, 1973) or carbamylcholine (Karlin & Cowburn, 1973) all of which exhibit a preferential affinity for the receptor site, or a salt gradient (Schmidt & Raftery, 1972). Purification factors of 200 fold were reported by Meunier et al. (1974) coupled with yields of 30%.

Purification to homogeneity was achieved by additional steps such as sucrose density gradient ultracentrifugation (Lindstrom & Patrick, 1974; Meunier & Changeux, 1973; Meunier et al, 1974) DEAE chromatography (Klett et al., 1973) or electrophoresis (Eldefrawi & Eldefrawi, 1973).

Meunier et al., in 1972 showed by gel filtration on Sepharose 6B that the receptor protein isolated from eel electric tissue, solubilized in deoxycholate and labelled with tritiated cobra toxin, had a molecular weight of 540,000 daltons. Dialysis of the extract against a solution of sodium dodecyl sulphate (SDS) caused disaggregation of the receptor-toxin complex, without the toxin itself dissociating. Results obtained from gel electrophoresis suggested

a molecular weight for each complex of 55,000 daltons. Assuming that a cobra toxin molecule was associated with each complex, the sub unit molecular weight was calculated to be 48,000. A degree of uncertainty was introduced however, when it was demonstrated that the free toxin in the presence of detergent behaves as though its molecular weight was 25,000 daltons. In 1972, Reiter et al.using a combination of affinity labelling and gel electrophoresis showed that the molecular weight of the complex in the presence of SDS was 42,000, which corresponded closely to the values obtained by Meunier et al., (1972). The large scale purification of the nAChR has been carried out by Sobel et al. (1977) using electric tissue from Torpedo marmorata, the method being basically an improvement on that of Cohen (1972). Four main polypeptide components were demonstrated to be present with molecular weights in the range 40,000 - 60,000 daltons. When the receptor-rich membrane fragments were labelled with tritiated MPTA,the use of polyacrylamide gel electrophoresis in SDS solution showed that in agreement with Karlin and Cowburn (1973) only the 40,000 dalton band was radioactive. If the electrophoretic method of Ames (1974) rather than Anderson et al.(1972) was used however, only three bands were observed, with no separation of the 40,000 and 43,000 dalton bands.

It is now generally accepted that only the 40,000 - 43,000 dalton band carries the binding site for ACh. Indeed, Valderama et al.,(1976) have shown that immunological cross-reactivity between receptor proteins from Electrophorus and Torpedo were due only to the 40,000 dalton chain (see also Meunier et al., 1974).

Patrick et al. (1975) have suggested that components of less than 48,000 daltons are artefacts resulting from proteolytic degradation, but Sobel and Changeux (1977) and Hucho et al. (1976) have shown that the 40,000 dalton band still occurs even in the presence of the proteolysis inhibitor phenylmethylsulphonylfluoride (PMSF). When PMSF was incorporated, the AChR-rich membranes migrated on a sucrose gradient exactly as they did in its absence. Conversely, when 1 mM ethylenediaminetetracetic acid (EDTA) was used, many proteins including the Na^+/K^+ ATPase and AChE migrated with the receptor-rich membranes, resulting in a decrease of specific activity.

Several bands have been obtained using SDS polyacrylamide gel electrophoresis with pure receptor material from many species of electric fish (Table 1). In two instances, (Raftery et al, 1976; and Karlin et al., 1976), a constant ratio between the four bands present was reported, with stoichiometries of 4:2:1:1. The AChR from Torpedo californica purified in its detergent solubilized form, also gave four polypeptide bands under the same conditions with molecular weights of 40,000; 50,000; 60,000 and 65,000 daltons, the latter band was assigned the specific function of binding small cholinergic ligands and snake α neurotoxins (see Weill et al., 1974; Hucho et al., 1976).

Sobel et al. (1977b) obtained bands of 40,000 and 43,000 daltons from Torpedo marmorata which, after dissolution in Triton X-100 gave a receptor protein with a sedimentation coefficient of 9S, and which bound tritiated MPTA. After further purification steps the 40,000 dalton band labelled in this way was the only one to be found after polyacrylamide gel electrophoresis in SDS solution. In the

absence of detergent however, the 43,000 dalton band was shown to bind specifically those reagents which are known to interact with the ionophore, particularly the frog venom histrionicotoxin.

Species	Sub unit MW (SDS-PAGE)	References
Torpedo marmorata	42,000	Potter (1973)
	40,000, 43,000, 50,000 & 60,000	Sobel et al. (1977)
	45,000 - 50,000	Heilbroon & Mattsson (1974)
Electrophorus electricus	52,000 (plus α toxin)	Meunier et al. (1972)
	42,000	Reiter et al. (1972)
Torpedo californica	26,000, 35,000 &42,000	Raftery et al. (1971) Raftery, Schmidt & Clark, (1972); Schmidt & Raftery (1973) Raftery (1973)
Torpedo nobiliana	34,000,36,000,39,000, 44,000 & >70,000	Ong & Brady (1974) Lindstrom et al. (1979) Lindstrom et al. (1980).
Narcine japonica	33,000,38,000,43,000 & 51,000	Ishikawa et al. (1980)

Table 1. A comparison of the Sub-Unit Molecular Weights of Receptor Material from Various Species of Electric Fish.

2.4 Reconstituion of the Purified Receptor

Physical reintegration of the receptor into a membrane environment has been successfully carried out by a number of investigators in order to determine if its performance matches the *in vivo* function. Parisi *et al.* (1971; 1972) reported responses of receptors to ACh in phospholipid membranes by increases in conductance. Using chloroform/methanol extracted proteolipid from *Electrophorus electricus*, they showed that ACh antagonists generally blocked the response to ACh but frequently themselves caused conductance changes. The application of αbgt caused a slow increase in conductance followed by rupture of the membrane. All such effects occurred only when the receptor proteolipid was added to the membrane. Similar results were obtained by Ochoa *et al.* (1972). The interpretation of data like this was complicated however by the results of del Castillo *et al.* (1966; 1967) who found that antigen-antibody reactions occurring in lipid membranes also caused marked conductance changes, as did the application of cholinergic drugs to AChE in a lipid film environment (see also Lenzinger & Schneider, 1972; Jain *et al.*, 1973).

Hazelbauer & Changeux (1974) provided the first real demonstration that the receptor could under certain conditions be reintegrated into a membrane in a functional form, while Vasquez *et al.* (1971) had employed electron microscopy to observe ultrastructural changes in lipid films, while Briley & Changeux (1976) used fluorescence probes such as quinacrine.

Lindstrom *et al.* (1980) used five purification methods, and incorporated the resulting receptor preparations into artificial membranes

using cholate dialysis. They showed that AChR reconstitution had developed into an easily reproducible process, and that as long as the cation channel was protected by cholate-lipid mixtures, most purification methods would produce an equally effective reconstituted AChR.

2.5 Immunological Studies

Another approach taken to ensure that proteins purified from electric tissue really were the physiological receptor, was to raise antibodies against them and assay to see whether such antibodies reacted with the receptors *in situ*. The first evidence for the validity of this approach was provided,albeit unintentionally, by Patrick & Lindstrom (1973) who injected purified *Torpedo* receptor preparations into rabbits in order to induce the formation of anti-AChR antibodies. During the course of the experiment however, the animals developed a flaccid paralysis similar to that seen in the human disease myasthenia gravis, and asphyxiated if they were not killed first. The electromyogram performed on muscle from the immunized rabbits showed signs of fatigue, and their serum contained immunoglobulins which precipitated the purified *Torpedo* receptor protein (Patrick & Lindstrom, 1973; Sugiyama *et al.*, 1973; Patrick *et al.*, 1973). The flaccid paralysis was attributed to an autoimmune response directed against endplate cholinergic receptors, and these results were confirmed by other investigators using different animals (Heilbronn & Mattsson, 1974; Heilbronn *et al.*, 1975; Tarrab-Hazdai *et al.*, 1975; Green *et al.*, 1975).

The serum from the immunized rabbits precipitated the receptor protein from Electrophorus in both its crude extract and purified forms obtained with both snake α neurotoxin and cholinergic spacer arm columns, and also precipitated Torpedo receptor protein (Sugiyama et al., 1973). Precipitation of the toxin-receptor complex required a much larger quantity of anti-receptor serum than the free receptor (Sugiyama et al., 1973; Patrick et al., 1973). Binding of the toxin therefore appeared to interfere in the reaction of the immunoglobulins present in the rabbit serum to antigenic determinants that were part of the receptor site. Patrick et al. (1973) have also shown that several reversible cholinergic ligands interfere in a similar fashion in the reaction of some antisera (but see Sugiyama, 1973).

Antibodies directed against the globular forms of AChE did not precipitate the cholinergic receptor protein prepared from the same tissue. Conversely anti-AChR antiserum showed little reaction with AChE. No cross-reaction could therefore be demonstrated between two closely related membrane proteins, although Sugiyama et al. (1973) have demonstrated such cross reactivity between nicotinic acetylcholine receptors from non-related zoological groups. Clearly the antibodies were directed against a protein which bound both cholinergic ligands and snake α toxins and so by definition was the nAChR. Sera raised in rabbits immunized against receptor protein purified by either method of affinity chromatography (Sugiyama et al., 1973; Patrick et al., 1973) blocked the response of the isolated electroplaque to bath applied carbamylcholine, while serum from non-immunized rabbits at the same dilution showed no effect.

This animal model of the human disease has been termed experimental autoimmune myasthenia gravis (EAMG) and detailed studies by Lindstrom, Lennon and colleagues have elucidated the pathogenesis of the disease (Lennon et al., 1975; 1976; Seybold et al., 1976; Lindstrom et al., 1976a). EAMG has now been induced in rabbits, rats, guinea pigs, monkeys, goats and dogs (Lennon, 1975; Lennon et al., 1976; Lambert et al., 1976).

Animals with EAMG showed striking similarities to the clinical symptoms of the human disease. At low and rapid rates of motor nerve stimulation there was a decremental response of muscle, and repetitive stimulation was followed by postactivation facilitation and exhaustion. Acetylcholinesterase inhibitors were shown by Seybold et al. (1976) to "repair" the electrophysiological defect, the amplitude of the miniature end plate potentials being reduced, with a normal amount of ACh released (Lambert et al., 1976). The lesions at the motor end-plates in rats with EAMG showed a strong resemblance to those seen in human myasthenics. Several groups have reported an immune response against nAChR in myasthenics, using a variety of techniques (Almon et al., 1974; Appel et al., 1975; Bender et al., 1975; Lindstrom et al., 1976b).

Lindstrom in his 1979 review, mentions the fact that results available from a number of researchers are consistent with the idea that anti-AChR antibodies bind to sites on the AChR other than those for ACh binding, and these antibodies when bound to some sites have an allosteric effect on AChR function. Antisera to electric organ AChR directly affects AChR function in electroplaque cells (Patrick

et al., 1973; Lindstrom et al., 1977; Karlin et al., 1978). Greater than 80% blockage of the depolarizing response of eel electric organs to applied carbamylcholine was blocked by pre-incubation with anti-AChR. Under these conditions, a large fraction of the AChR in electroplaque cells had antibodies bound, but there was little or no impairment of ^{125}I-toxin binding (Lindstrom et al., 1977).

2.6 Chemical Studies on the Isolated nAChR

The amino-acid composition for the purified receptor from both Electrophorus and Torpedo has been established and contains all the amino acids typical of globular proteins (Heilbronn et al., 1973; Eldefrawi & Eldefrawi, 1973; Meunier et al., 1974; Klett et al., 1973). The absence of tryptophan was reported by Klett et al. (1973), but this was challenged by others working in the same field and tryptophan was claimed to be present in the vicinity of the receptor site at a concentration of 2.5 moles percent. The amino acid composition of the most purified preparations of receptor protein from both species of fish were shown to be very similar, but differed significantly from that for AChE purified from the same tissue (Meunier et al., 1974; Ong & Brady, 1974; Eldefrawi & Eldefrawi, 1973; Rosenberry et al., 1972). Capaldi and Vanderkooi (1972)have stated that the receptor contains about 46% polar residues, a value considered typical for globular, water soluble proteins, and not reflecting the hydrophobic character of the receptor molecule, which may derive from an asymmetric distribution of these polar amino acids. Raftery et al., 1976, however,using Barrantes' (1975) method for the analysis of hydrophobicity has suggested a similarity between the receptor and known integral membrane proteins.

Purified preparations of receptor do not appear to possess any covalently bound phospholipid (Klett et al., 1973) but they do contain carbohydrates. Klett et al.(1973) reported that receptor from Electrophorus contained carbohydrate while Meunier et al, (1974) showed that it reacted with a variety of plant lectins. Concanavalin A (Con A) which is known to react with mannosyl residues precipitated the purified protein and α-methyl D-mannose dissociated the Con A-receptor complex. The receptor also bound lectins obtained from Phaseolus vulgaris (N-acetyl-D-galactosamine); Lens culinaris (D-galactose) and Triticum vulgare (di-N-acetylchitobiose). N-acetyl-D-glucosamine has been detected in receptor preparations from Torpedo (Michaelson et al., 1974; Moore et al., 1974). Preparations from this source also contained neutral sugars (5% by weight), with mannose, galactose and glucose in the ratio 8:2:1 (Raftery et al., 1975). In a similar preparation, Heilbronn (1975) reported a ratio of 8:1.8:0.2. Raftery et al. (1973) considered that the carbohydrate moiety of the receptor might constitute up to 20% of the total mass of the purified preparation from Torpedo, although Meunier et al. have proposed a smaller proportion (see also Heilbronn & Mattsson, 1975).

Recently, Ishikawa, Yoshida & Tamiya (1980) have published a value of 18% carbohydrate by weight found in receptor preparations from the electric tissue of the Japanese ray Narke japonica. The following table shows the published carbohydrate contents of pure nAChR isolated from the electric organs of various species of electric fish.

Species	% Carbohydrate	References
Electrophorus electricus	present	Meunier et al., (1974)
Torpedo marmorata	3-4	Heilbronn & Mattson (1975)
Torpedo californica	5	Michaelson et al. (1974)
Torpedo nobiliana	3-8	Moore et al. (1974)
Narcine japonica	18	Ishikawa et al. (1980)

Table 2. A Comparison of the Percentage Carbohydrate by Weight Obtained from Pure AChR isolated from the Electric Organs of Various Species of Electric Fish.

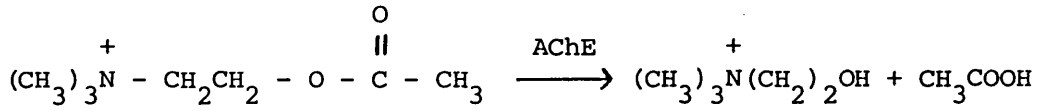
Sialic acids although common in many membrane glycoproteins have never been convincingly demonstrated to be part of the nAChR from either species of fish (Heilbronn, 1975), although it is interesting to note that Werner et al.(1978) demonstrated the presence of N-acetyl-neuraminic acid, together with mannose, fucose N-acetylglucosamine and galactose, in preparations of acetylcholinesterase from Torpedo.

On a cautionary note, Eldefrawi and Eldefrawi (1973) have discussed the possibility that contamination from agarose columns used during purification might mask the presence of hexosamines, and artificially boost the quantities of neutral sugar found. The bulk of published evidence however points to the fact that the nAChR from electric organ tissue is, like many other membrane proteins, a glycoprotein.

2.7 Acetylcholinesterase

The main role for acetylcholinesterase (AChE) is believed to be the termination of nervous impulse transmission by hydrolysis of

acetylcholine:



(Acetylcholine) (choline) (acetic acid)

Fractionation and cytochemical studies indicate that AChE is associated with the surface membrane of the excitable cell, although its precise relationship to the excitable membrane is still not totally clear. Some studies have indicated that the enzyme is not tightly associated with the plasma membrane, while Silman & Karlin (1967) have shown that much of the molecule is readily solubilized at high ionic strength under conditions which do not lead to any appreciable solubilization of the AChR (Karlin & Cowburn, 1973). A number of researchers have demonstrated that the enzyme is easily solubilized by limited protease treatment (Massoulie et al., 1970; Dudai & Silman, 1974b; Taylor et al., 1974). Hall (1973) showed that in skeletal muscle too, significant amounts of AChE could be solubilized without the use of detergents.

The electric tissue of Electrophorus electricus provided the source from which AChE was originally purified and characterized (Kremzner & Wilson, 1964), and it was subsequently shown that the molecule was an 11s globular protein in tetrameric form, possessing four similar active-site bearing subunits each of molecular weight 80,000 daltons (for reviews see Rosenberry, 1975; Silman, 1976). The tetramer is actually composed of two dimers of molecular weight 160,000 daltons which are linked together by disulphide bonds. (Froede & Wilson, 1970; Dudai & Silman, 1974; Rosenberry et al., 1974).

The 11s form of the enzyme does not occur in fresh tissue but rather in three forms: with sedimentation coefficients of 18s, 14s, and 9s (Massoulie & Rieger, 1969) which are converted to the 11s form as a consequence of proteolysis (Massoulie et al., 1970; Dudai et al., 1972). It has been demonstrated by a number of investigators that all three forms from fresh tissue are asymmetric structures containing a multi-subunit head and elongated tail (Dudai et al., 1973; Rieger et al., 1973; Bon et al., 1973; 1976).

2.8 The Nicotinic Acetylcholine Receptor (n AChR)

The receptor protein isolated from a variety of sources has been shown to contain carbohydrate as demonstrated by lectin binding (Meunier et al., 1974; Brockes & Hall, 1975; Mittag et al., 1978; Boulter & Patrick, 1979; Wonnacott et al., 1980b) and chemical analysis (Mattsson & Heilbronn, 1975; Raftery et al., 1976; see also Weinberg & Hall, 1979).

It has been demonstrated by Neubig (1979) and Vandlen et al. (1979) that the 40,000, 50,000 and 66,000 dalton subunits, but not the 43,000 dalton subunit can be stained on polyacrylamide gels by carbohydrate specific stains, or labelled with ^{125}I-Con A (Wennogle and Changeux, 1980) the carbohydrate moieties being found systematically exposed to the outer face of the membrane. The 40,000 dalton subunit which contains all or part of the ACh binding site, as evidenced by the selective binding of ^{3}H-MBTA contains carbohydrate, but this is probably not involved in either the binding of ACh or its agonists (Wennogle & Changeux, 1980; Wonnacott et al., 1980a).

When purified nAChR was incubated with a mixture of protease-free glycosidases, there was extensive removal of carbohydrate as demonstrated by a 70% reduction in Con A binding capacity but no significant decrease in the antigenicity of the carbohydrate depleted receptor (Wonnacott et al., 1980b). These results showed little evidence for the role of carbohydrate groups as antigenic determinants on the receptor protein, and tend to indicate that antibodies to Torpedo receptor are directed primarily at other sites on the molecule. Further treatment with periodate again destroyed carbohydrate by diol cleavage but did not alter the antigenicity of the receptor molecule.

Wennogle and Changeux (1980) have published a model of the topological arrangement of the four main polypeptide chains in the membrane of Torpedo electroplax, based on their studies using selective proteolysis. This model depicts the 40,000, 50,000 and 66,000 dalton subunits as spanning the bilayer and possessing carbohydrate, while the 43,000 dalton subunit was devoid of carbohydrate and buried in the interior of the membrane.

3. TECHNIQUES USED IN THE ISOLATION AND CHARACTERIZATION OF MEMBRANE PROTEINS

The range of isolation techniques which may be employed for membrane proteins depends very much on the degree of biological activity which is required to be preserved. If the aim is for a structural and chemical characterization then the range is wide because the possibility of denaturation is not a concern. Conversely, if the biological activity of the isolated protein is to be retained, then the choice of techniques is obviously limited to those which do not lead to a loss of activity. As yet the choice available does not approach that for soluble proteins and so it is usually necessary to ensure that the starting material is as pure and homogeneous as possible.

Whether or not the protein is peripheral or integral has a bearing on the isolation methods to be employed. Helenius and Simons (1977) have devised a method of charge-shift electrophoresis which is capable of discriminating between the two on the basis of the binding of non-ionic detergents such as Triton X-100. This method was adapted by Bhakdi et al. (1977) to include two dimensional and immuno-electrophoresis which gave better resolution of proteins in complex mixtures.

3.1 Solubilization of Membrane Proteins

(i) Peripheral Membrane Proteins

The term "peripheral" has been applied to those membrane proteins which may be solubilized from the membrane without disruption or solubilization of the lipid bilayer (see Table 3). Once isolated in a lipid-free form they can usually be separated using those

techniques applicable to soluble proteins (Jacoby, 1971).

Reagent	Membrane	Reference
NaCl (1M solution)	Red blood cell	Tanner & Boxer (1972)
EDTA (1mM solution)	Sarcoplasmic reticulum	Thorley-Lawson & Green (1973)
Acetic Acid (10%)	Red blood cell	Schubert (1973)
NaOH (0.1N solution)	Red blood cell	Steck & Yu (1973)
Chaotropic ions	Red blood cell	Hatefi & Hanstein (1974)

Table 3. Reagents used to solubilize peripheral membrane proteins.

All chaotropic salts will cause denaturation and solubilization of the membrane if used at sufficiently high concentrations, but they can be selectively effective at low concentrations. Thus, lithium diiodosalicylate selectivity solubilized only the peripheral proteins of the red cell membrane at a concentration of 40 mM (Steck & Yu, 1973) whereas the use of a 0.3 M solution resulted in complete solubilization of the membrane (Marchesi & Andrews, 1971). A number of investigators have also demonstrated that both urea and guanidine hydrochloride are selective in their solubilization of peripheral membrane proteins (Juliano & Rothstein, 1971; Maddy & Kelly, 1971a; Steck, 1972). However, Steck and Yu (1973) have shown that the same result may be obtained using much milder reagents. Protein-modifying reagents such as organomercurials and acid anhydrides may be employed to the same effect, but detrimental effects on biological activity could result (Carter, 1973; Steck & Yu, 1973; Lundahl, 1975; MacLennan et al., 1965).

(ii) Integral Proteins

The amphiphilic nature of these molecules inevitably causes problems in their isolation and purification. Because they are normally associated with a phospholipid bilayer, those very interactions which enable them to be located in the membrane tend to cause instability in both aqueous and organic solvents. When exposed to an aqueous medium, self association at the hydrophobic surfaces minimizes contact while the hydrophilic surfaces try to maximize the area in contact with water. The thermodynamically stable state which results from trying to satisfy both criteria usually leads to aggregation.

It is likely that totally hydrophobic proteins exist which are *in vivo* totally immersed in the phospholipid bilayer, such as the cholinergic proteolipid isolated by De Robertis *et al.* (1971) and those from myelin (Folch-Pi & Stoffyn, 1972; Cattel *et al.*, 1970). Proteins such as these would be soluble in mixtures of solvents such as chloroform: methanol, but the majority are not and will undergo denaturation if exposed to them. The inherent limitations of a tendency towards associative behaviour and non-solubility in either polar or non-polar solvents, led to the search for alternative methods of extraction because of the difficulties experienced in the application of these techniques which were originally developed for water soluble proteins.

An answer to this problem was found in studies on the interactions of detergents with membranes and their use in the extraction of integral membrane proteins (Helenius & Simons, 1975; Tanford & Reynolds, 1976). When complexed with a detergent a membrane protein

exists in a state which parallels that of the intact membrane. It is in this type of environment that it functions normally, and so it follows that here the protein will exist in its most stable form. A micellar structure is formed by the detergent which simulates the phospholipid bilayer environment, conferring solubility in an aqueous environment. Three main types of detergent have been employed in the routine isolation of integral membrane proteins:-

(a) non-ionic - these are generally composed of molecules which possess polar polyoxyethylene head groups and have molecular weights in the 50,000 - 100,000 dalton range, with low critical micellar concentrations. Non-ionic detergents do not bind to soluble proteins unless they contain hydrophobic sites, in which case detergent will be bound easily and with great efficiency (Helenius & Simons, 1972; Makino et al., 1975). Some of the factors involved in choosing a suitable detergent for membrane solubilization have been considered in detail by Tanford and Reynolds (1976). Where the retention of biological activity is required, the non-ionic detergents such as Tween 80; Lubrol WX; Nonidet P-40 and Triton X-100 (Clarke, 1975) are to be preferred.

(b) ionic - these generally possess strongly acidic or basic polar head groups such as SO_4^- or NH_4^+, with smaller micelles and a relatively higher critical micellar concentration than non-ionic detergents. Ionic detergents will bind to soluble proteins in a cooperative manner which results in unfolding of the polypeptide chain. Most proteins will bind sodium dodecyl sulphate (Grefrath & Reynolds, 1974) at approximately 1.4 g per gram of protein (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970). Some

investigators have shown that in a few cases, solubilization with ionic detergents does not result in a loss of biological activity (Salton & Netchey, 1965; Crane & Lampen, 1970; Spatz & Strittmater, 1971). However, Medzhiradsky et al. (1967) demonstrated that denaturation invariably occurs when such detergents are used.

(c) Bile salts - these molecules differ from both ionic and non-ionic detergents in that they do not form disc-shaped micelles, but rather small aggregates composed of a number of monomers (Cary & Small, 1972). Because they contain carboxyl groups, bile salts possess properties which vary at pH values around that of the pKa. Bile salts are fairly safe with regard to the preservation of biological activity during solubilization. The stages in the solubilization of membranes by all types of detergent have been studied by Helenius & Simons (1975).

Both ion-exchange and gel filtration are possible in the presence of non-ionic detergents and bile salts. Klett et al. (1973) have shown that hydroxylapatite may be used in the isolation of the nAChR using Tween 80. The range of techniques available for use in the presence of ionic detergents is limited however. Gel filtration in SDS has been applied to the purffication of red cell membrane proteins (Tanner & Boxer, 1972; Ho & Guidotti,1975; Tanner et al., 1976). Preparative SDS electrophoresis has been successful in some cases (Tanner & Boxer, 1972; Chai & Foulds, 1977) but poor recoveries and the limited amounts of material processed have proved major disadvantages.

(iii) Organic Solvents

Some membrane proteins are soluble in organic solvents and the term "proteolipid" was coined for them by Folch and Lees (1951). The myelin and cholinergic receptor proteolipids which are soluble in chloroform:methanol mixtures are two well known examples. (Shooter & Einstein, 1971; Folch-Pi & Stoffyn, 1972; De Robertis 1971; Moscarello, 1976).

A number of other membrane proteins will also partition into a butanol or chloroform phase at pH 7. The C_{55} isoprenoid alcohol phosphokinase from Staphylococcus aureus (Sanderman & Strominger, 1972), the dicyclohexylcarbodiimide reactive subunit of the Escherichia coli ATPase (Altendorf et al., 1977), proteolipids from the sarcoplasmic reticulum Ca^{2+} ATPase (MacLennan, 1974) and the mitochondrial ATPase (Tzagoloff et al., 1973) are all examples of such proteins.

The biological activity of any given membrane protein is not necessarily lost if after extraction into an organic solvent phase, the solvent is subsequently removed. Montal (1976) and Darszon et al. (1977) have suggested that many membrane proteins will behave as proteolipids if the protein complex is neutralized by suitable counterions.

Despite the highly polar nature of glycosylated membrane proteins, there are those which may be solubilized in organic solvents. Fletcher et al. (1977) demonstrated that a thymocyte plasma membrane glycoprotein which contained 30% - 40% carbohydrate, was purified more effectively by extracting with 75% ethanol than by the lithium diiodo-

salicylate method more commonly used. The infectious mononucleosis heterophile antigen found on bovine red cells, is soluble in both hot 75% ethanol (Fletcher & Woolfold, 1971) and aqueous chloroform: methanol mixtures (Merrick et al., 1977). This molecule is a glycoprotein of approximate molecular weight 26,000 daltons, and contains about 10% carbohydrate. Hamaguchi & Cleeve (1972) have shown that this molecule in common with the sialoglycoproteins of human red cells, may be substantially purified by partition with the aqueous phase during solvent extraction of red cell ghosts. Selective solubilization of the major human red cell glycoproteins may also be effected by using butanol extraction. Maddy (1966) has shown that extraction of red cell ghosts in this manner at low ionic strength results in the solubilization of most of the membrane protein in the aqueous phase, but if a high salt concentration is used the sialoglycoproteins only are recovered from the aqueous phase (Anstee & Tanner, 1974).

Both pyridine and phenol have been used for the extraction of red cell and milk fat globule membranes, with each solvent being effective in selectively removing sialoglycoproteins into the aqueous phase (Blumenfeld et al., 1970; Tanner & Boxer, 1972; Klenk & Uhlenbruck, 1960; Kathan et al.,1961; Springer et al., 1966; Newman et al., 1976).

The further fractionation of membrane proteins solubilized using organic solvents has been pursued by few investigators, however, De Robertis and his colleagues have pioneered the purification of the cholinergic proteolipid after chromatography on Sephadex LH-20

(De Robertis et al., 1971) while Sandermann and Strominger (1972) combined fractional solubilization using methanol:butanol mixtures, with DEAE chromatography and Sephadex G-50 gel filtration in their isolation of the C_{55} isoprenoid alcohol phosphokinase from Staphylococcus aureus. A combination of these two techniques has been employed using chloroform:methanol mixtures by a number of investigators (Fillingame, 1976; Altendorf, 1977; Altendorf et al., 1977; Merrick et al., 1977). Aprotic solvents such as dimethylformamide (Kohl & Sandermann, 1977) and acidic solvents like 2-chloroethanol, acetic acid and formic acid can completely solubilize membranes, but the range of techniques available for further purification in such solvents is somewhat limited (Zahler, 1974; Schubert, 1973). However, Schubert (1977) used preparative electrophoresis in 90% acetic acid to purify the major red cell membrane protein.

(iv) Membrane Proteolysis

Many membranes contain proteases which may be activated by a wide variety of conditions, but especially by those prevalent in the commonly used extraction procedures (Morrison & Neurath, 1953; Moore et al., 1970; Tanner & Boxer, 1972; Tokes & Chambers, 1975; King & Morrison, 1977). The occurrence of proteolysis during such procedures is readily detected by employing SDS-PAGE, whereupon low molecular weight bands may be detected and there is a general loss of sharpness seen with stained protein bands. The background staining to the gels is also difficult to remove, particularly where a stain such as Coomassie Blue is used.

The proteolysis of native material is not temperature dependent,

and Tanner and Gray (1971) have demonstrated the occurrence of proteolysis in red cell membranes during storage at -20°C. The presence of high salt concentrations is particularly detrimental. A number of protease inhibitors have been employed during the early stages of membrane extraction and solubilization in order to reduce the effects of proteolysis. Of the many that are available, the most commonly used have been diisopropylfluorophosphate, phenylmethylsulphonylfluoride, and ethylene diamine tetra acetic acid (Tanner & Boxer, 1972; Dolly & Barnard, 1977; Sobel & Changeux, 1977). Those extraction proceudres which utilize very high salt concentrations (Reisfield & Pellegrino, 1972) are only effective because of the stimulation of autoproteolysis which results in the release of soluble protein fragments from the membrane (Mann, 1972).

Proteases which exhibit a broad substrate specificity, such as trypsin, pronase, papain, collagenase and chymotrypsin, have been extensively used for the deliberate degradation of membrane glycoproteins with the release of a mixture of glycopeptides which were subsequently subjected to a wide range of fractionation and purification procedures (Pepper & Jamieson, 1969; 1970; Barber & Jamieson, 1971; Winzler, 1969; Phillips, 1972; Winzler et al., 1967; Harrison et al., 1975; Harrison, 1975; Harrison et al., 1978; Farrar & Harrison, 1978; Berg, 1974; Rodbell, 1964; Wennogle & Changeux, 1980). A range of membranes have been treated in this way including platelets; basement membrane, lymphocytes, tumour cells, milk fat globules, electroplax membranes from various species of electric fish, and the human red cell upon which the bulk of such research was performed.

3.2 Analytical Techniques used for the Characterization of Membrane Proteins

(i) Polyacrylamide Gel Electrophoresis (PAGE)

The analysis of proteins by electrophoresis in a gel matrix has gained importance over the last decade particularly with the development of discontinuous electrophoresis in acrylamide gels (Raymond & Weintraub, 1959; Davis, 1964; Ornstein, 1964). In these systems it is possible by adjustment of the pH, ionic strength and temperature of the buffer system used to separate proteins that differ only slightly in isoionic points and molecular size (Hendrik & Smith, 1968). The advantages of using polyacrylamide gels stem from the fact that they are inert as far as proteins are concerned and they can be prepared over a wide range of parameters to suit the spectrum of protein molecular weights for which they act as molecular sieves. The pore sizes may be varied by altering the proportions of acrylamide and methylene bis acrylamide monomers so that it is possible to produce gels with high concentrations for low molecular weight proteins, and low (3%) concentrations for separating molecules of several million daltons (Peacock & Dingman, 1968). The procedure has been specifically adapted for the separation of membrane proteins by the incorporation into the buffers of reagents which keep the proteins in solution. Thus there are examples of systems containing urea (Schneidermann, 1965; Zwaal & Van Deenen, 1968; Neville, 1967) buffers of varying pH (Maddy & Kelly, 1971) phenol-acetic acid-water (Takayama et al., 1966; Takayama & Stoner, 1969; Ray & Marinetti, 1971) chloral hydrate (Ballou et al., 1974; Ballou & Smithies, 1977), non-ionic detergents (Dulaney & Touster, 1970; Scandella & Dornberg, 1971; Dewald et al.,1974; Fries, 1976), bile salts (Dulaney & Touster, 1970) and ionic detergents (Weber

& Osborn, 1969).

However, unless protein dissociating reagents are used in conjunction with this electrophoretic technique, it is not possible to be certain that the individual bands obtained are not in fact composed of heterogeneous mixtures and protein aggregates. An authoritative review on electrophoretic techniques which discusses this problem has been published by Dunn and Maddy (1976).

Because the use of non-ionic detergents and bile salts solubilizes integral membrane proteins but does not necessarily dissociate them into individual polypeptide chains, the use of the ionic detergent sodium dodecyl sulphate (SDS) has become almost universal in membrane protein research because of its potent solubilizing and dissociating properties. Gel electrophoresis using SDS was introduced by Summers et al. in 1965. It was shown by a number of investigators that separations were achieved in which a clear relationship existed between the migration of the protein component and the logarithm of its molecular weight (Shapiro et al., 1967; Weber & Osborn, 1969; Dunker & Rueckert, 1969). When used for soluble proteins this method is fairly reliable for those above a certain critical molecular weight, but below this value the relationship does not hold (Dunker & Rueckert, 1969).

Reynolds and Tanford (1970a, b) investigated the binding of SDS to soluble proteins and showed that, provided that these proteins were treated to reduce their disulphide bonds to sulphydryl groups, then massive detergent binding (approximately 1.4 g SDS per g protein) occurred accompanied by structural changes which gave the SDS-protein

complexes a prolate ellipsoid shape, the length of which depended on the polypeptide molecular weight. The bound SDS swamped all the protein charges contributed by ω-amino and ω-carboxyl groups, such that the detergent-protein complexes all possessed the same charge to mass ratio. Thus as all the complexes should be of an equivalent shape, separation was achieved on a strict molecular weight basis because of the sieving action of the acrylamide gel. However, Nelson (1971) has demonstrated that basic proteins bind more, and acidic proteins bind less SDS than average so it would seem that amino acid composition influences the degree of detergent binding. When the SDS gel system is applied to the separation of integral membrane proteins, there is reason to believe that the apparent molecular weights obtained are not always correct. Because, as previously outlined the hydrophobic regions of these proteins bind detergent in a micellar fashion and as this binding differs considerably from that shown by soluble proteins, the observed molecular weights are liable to be incorrect because soluble proteins are generally used to calibrate the molecular weight range required for the technique.

Many integral membrane proteins are in fact glycoproteins and these molecules tend to show anomalous mobility effects. Bretscher (1971b) has shown that the major sialoglycoprotein from human red cells behaves in this way with its apparent molecular weight dependent upon the acrylamide concentration of the gel. These anomalies indicate that the molecular weights of integral membrane proteins should be treated as apparent molecular weights only (Tanford & Reynolds, 1976).

A number of variations to the SDS system have been described (Weber & Osborn, 1969; Tanner & Gray, 1971). The tris-acetate buffer system of Fairbanks et al.(1971) is widely used and has been employed by Juliano and Behar-Bannelier (1975) who claimed that aggregation was avoided and consistent and reliable analyses of high molecular weight glycoproteins achieved. Discontinuous gel systems offer the advantage of high resolution for the more rapidly migrating membrane species, having a band sharpening effect (Laemmli & Favre, 1973; Neville & Glossman, 1974).

Another variation of the standard electrophoretic system uses polyacrylamide gels with a linear concentration gradient. Using this system, proteins with molecular weights greater than 50,000 daltons migrate at a progressively slower rate as the run proceeds. Because, in acrylamide concentration gradients, the front of a protein band is always in a higher acrylamide concentration than the rear, it has a lower mobility and so sharpening occurs (Knufermann et al., 1975).

(ii) Isoelectric Focusing

This method utilizes the electrophoretic migration of proteins in a pH gradient such that under equilibrium conditions they congregate at those pH zones which correspond to their isoelectric points, and has proved to be a very high resolution technique for the separation of soluble proteins (Vesterberg & Svenson, 1966; Catsimpoolas, 1973). This method was adapted by Adweh et al.(1968) for use in acrylamide gels, and has been used by a number of investigators with membrane proteins after the incorporation of non-ionic detergent

or urea into the gel (Merz et al., 1972; Miner & Heston, 1972; Bhakdi et al., 1974; Cook, 1976).

(iii) Immunochemical Techniques

The immunogenicity of proteins depends to a large extent on certain of their molecular properties (Sela, 1969; Crumpton, 1974) and while Gill et al. (1967) proposed that a higher content of hydrophobic residues should increase the immunogenicity, Atassi (1975) found that all the antigenic determinants in sperm whale myoglobin were hydrophilic in nature.

Membrane proteins have been found to be immunogenic both when exposed on the membrane surface (Blomberg & Perlmann, 1971; Bjerrum & Lundahl, 1974; Bock et al., 1974; Johansson & Hjerten, 1974). and also after solubilization (Masters et al., 1971; Maretzki et al., 1973; Papermaster et al., 1975) . Many membrane proteins are immunogenic and will induce antibody formation under the right conditions (Bjerrum, 1977). This has been clearly demonstrated with the human red cell membrane solubilized using non-ionic detergent (Bjerrum & Lundahl, 1974; Bjerrum et al., 1976) where the majority of the polypeotides were precipitated using rabbit antiserum to membrane preparations. It is naturally difficult to demonstrate that hydrophobic regions of membrane proteins are immunogenic because of their position within the lipid bilayer. Even after solubilization detergent molecules will bind specifically to these regions and so shield them from contact with antibodies. The production of antibodies is also dependent on the conformational state of the membrane

protein (Bjerrum et al., 1976; Bhakdi et al., 1976; Smith & Shapiro, 1974; Neeman et al., 1972).

Many animals have been used for immunization, but rabbits seem to be particularly suitable and have been used by many investigators (Bock et al., 1974; Johansson & Hjerten, 1974; Maretzki et al., 1973; Masters et al., 1971; Papermaster et al., 1975; Bjerrum et al., 1976; Smith & Shapiro, 1974; Neeman et al., 1972; Louvard et al., 1975; Kahane & Razin, 1969; Bhadki et al., 1976; Owen & Smyth, 1976; Fukui et al., 1971) because they are relatively easy to keep for an extended period of time and bleed on a regular basis.

It can be argued that it is preferable to immunize with membrane fragments rather than a solubilized preparation because the detergents used in such procedures can lead to denaturation and a loss of antigenic material through selective solubilization. We know from the work of Bjerrum and his colleagues (Bjerrum & Bog-Hansen, 1975) that membrane bound proteases are liberated during solubilization procedures, and this would render membrane material more likely to be degraded by non-specific proteolysis.

Precipitating antibodies for membrane proteins from red cells (Bjerrum & Lundahl, 1974) rat brain synaptosomal membranes (Bock et al., 1974) and milk fat globule membranes (Roast, 1975; Nielsen & Bjerrum, 1977) have all been described, and many investigators have used ionic, non-ionic and bile salt type detergents to solubilize membrane material for immunization (Bock et al., 1974; D'Amelio et al., 1963; Poyton & Schatz, 1975; Bhakdi et al., 1976; Owen &

Smyth, 1976).

A variety of methods are available for the solubilization of membrane proteins, as mentioned previously and reviewed by Razin (1972); Steck & Fox (1973); Helenius & Simons (1975); Maddy & Dunn (1976). Unfortunately not all of these are suitable for subsequent immunochemical analysis. The ideal solubilizing reagent for this type of procedure should release the proteins from their membrane and keep them in solution without alteration to their immunogenicity. Equally if the reagent forms part of the analytical procedure, there must be no inteference with the antibody binding site. Those procedures involving the manipulation of ionic strength,pH or treatment with chelating agents which are suitable for the solubilization of peripheral proteins, also permit immunochemical analysis (However, Bjerrum et al., 1974 have shown that the pH must be kept between 4.5 and 10.0).

Where integral membrane proteins are required to be solubilized, detergent treatment is necessary because extraction with organic solvents, chaotropic ions or chemical modification results in an interference with the antibody/antigen reaction. Indeed, solubilization with non-ionic detergents such as Triton X-100 or Lubrol WX seems to be the method of choice for the provision of components in a form suitable for immunochemical analysis. In aqueous solution (usually 1% v/v) such detergents extract and solubilize many membrane proteins (Helenius & Simons, 1975; Razin & Barash, 1969; Miller, 1970) and do not interfere with the antibody/antigen reaction (Bjerrum & Lundahl, 1973; Crumpton & Parkhouse, 1972).

Ionic detergents are not as suitable for immunochemical analysis because they are generally more denaturing in their action than non-ionic reagents (Helenius & Simons, 1975). Because of their negative charge they bind equally well to both hydrophilic and hydrophobic areas of proteins resulting in a conformational change to the polypeptide with a subsequent loss of immunogenicity. However, because as two groups of workers have shown (Helenius & Simons, 1975; Makino et al., 1973), the denaturing effect of ionic detergents is proportional to the concentration of free detergent, their use at suitably low concentrations may allow a degree of solubilization coupled with retention of immunological activity (Kahane & Razin, 1969; Fukui et al., 1971; Bjerrum et al., 1974; 1975; Poyton & Schatz, 1975; Nielsen & Bjerrum, 1975; Green et al., 1975; Owen & Smyth, 1976; Bhakdi et al., 1976). Subsequent immunochemical analysis in the presence of ionic detergents such as SDS is made difficult by its dissociating effect on the antibody/antigen complex, and the fact that electrophoretic procedures which depend on the surface charge of the protein could give rise to artefactual precipitation lines (D'Amelio et al., 1963; Nielsen & Bjerrum, 1975; Bjerrum et al., 1975; Green et al., 1975; Carey et al., 1975; Yu & Steck, 1975). The use of limited proteolysis with broad spectrum proteases has been successfully employed for the immunochemical analysis of histocompatibility antigens (Reisfield & Kahan, 1971; Davies, 1973) membrane enzymes (Louvard et al., 1975a, b; Takesue et al., 1973), tumour membrane antigens (Prat et al., 1975; Baldwin & Glaves, 1972; Baldwin et al., 1974). Raftell and Blomberg (1974) have commented that the extent of antigen release obtained by proteolytic degradation of membranes seemed generally inferior to that produced by non-ionic detergent extraction.

The use of chaotropic ions for membrane protein solubilization (Steck & Fox, 1973; Maddy & Dunn, 1976) is not compatible with immunochemical analysis because of their role as protein perturbents. Where reagents such as urea (Furthmayr & Timpl ,1970) lithium diiodosalicylate (Marchesi & Andrews, 1971; Nachman et al., 1973) pyridine (Furthmayr & Timpl, 1970; Howe et al., 1971; Anstee & Tanner, 1974) ethanol (Fletcher & Woolfold, 1971; 1972) phenol (Howe & Lee, 1969; Howe et al., 1971; Ebert et al., 1975) chloroform: methanol (Hamaguchi & Cleve, 1972; Wood et al., 1975) butanol (Adachi & Furnsawa, 1968; Poulik & Bron, 1969; Whiteside & Salton, 1970; Bron & Poulik, 1972) or formic acid (Poulik & Bron, 1969) are used those antigens which are resistant to denaturation and inactivation tend to be glycoproteins since the highly polar carbohydrate groups are not affected by non-polar reagents and the carbohydrate moieties often contain the antigenic determinants themselves.

(a) The antibody/antigen reaction

The initial discovery of infectious agents and associated mammalian defence mechanisms (Pasteur, 1876; Von Behring & Kitasato, 1890; Koch, 1891; Metchnikoff, 1892) was followed by the demonstration of serum antibodies (Kraus, 1897) and specific"serum factors" which neutralized the infectious agents (Ehrlich & Morgenroth, 1900). Arrhenius (1907) was the first to try to apply the Law of Mass Action to immunochemical analysis, although it is now clear that this does not apply to the complex secondary interactions involved in precipitate formation. The immunoprecipitation reaction was quantified by Heidelberger & Kendall (1929; 1935; Heidelberger, 1939), who used pneumococcal polysaccharide antigens which did not

interfere with antibody nitrogen determinations, and laid the theoretical basis for gel immunoprecipitation techniques. The combination of antibody and antigen may be considered as a reversible bimolecular reaction:

$$Ab + Ag \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AbAg$$

Ab = free antibody

Ag = free antigen

AbAg = antibody-antigen complex

(where k_1 and k_2 are the rate constants of the forward and backward reactions).

At equilibrium, the rates of association and dissociation are equal:

$$\frac{k_1}{k_2} = K = \frac{AbAg}{Ab \; Ag}$$

K = equilibrium constant

As the antigen concentration is increased, so the amount of the precipitating complex increases up to a certain point which is referred to as the "zone of antibody excess". No further increase in precipitated complex is noted above this point, so long as the same degree of equivalence continues to exist between antibody and antigen, and is termed the "zone of equivalence". With increasing antigen concentration above this point a state is reached where because of the antigen excess, an inhibition of complex formation occurs and there is a progressive decrease in the amount of the antibody-antigen complex precipitated (Figure 3).

Because of these results it was postulated that immunoprecipitate formation was due to a series of bimolecular reactions which

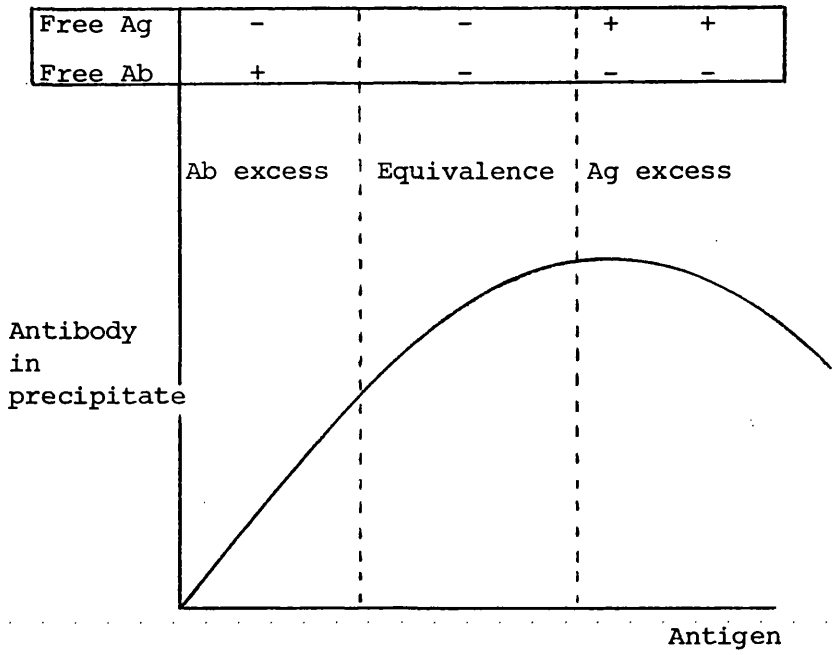


Figure 3. The Quantitative Precipitation Reaction

followed the Law of Mass Action:

$$Ab + Ag \rightleftharpoons Ab - Ag$$

$$Ab - Ag + Ab \rightleftharpoons (Ab)_2\text{-Ag antibody excess}$$

or $$Ab\text{-}Ag + Ab - Ag \rightleftharpoons (Ab)_2\text{-}(Ag)_2$$

The primary Ab-Ag complex was shown to be established within 10 seconds by Tengerdy & Small (1966) using light scattering and fluorescence quenching techniques. The Ab-Ag complex shows both temperature dependence between 0° - $4^{\circ}C$ for the rate of association, and also pH dependence (Kleinschmidt & Boyer, 1952). The immuno-precipitate is generally soluble above pH 10 and below pH 5.

(b) Ouchterlony double diffusion

Early in this century, Bechold (1905) showed that immunochemical analysis could be performed in gels. Quantitative methods for the identification of antigens and antibodies were introduced by

Nicolle et al. (1920) and Petrie (1932), and Sia and Chung (1932) adapted the method for their work on bacterial antigen identification. In 1946, Oudin published the physicochemical and mathematical basis of gel diffusion, this work being extended and modified by Elek (1948) and Ouchterlony (1949).

Gel immunodiffusion techniques rely on the fact that the diffusion of reactants creates concentration gradients in the gel, and as a result those concentration ratios of antibody-antigen which are suitable for precipitation occur in narrow zones. The precipitates become visible as arc-shaped opaque lines in the relatively transparent agarose gel.

The Ouchterlony technique (Elek, 1948; Ouchterlony, 1949; Ouchterlony & Nilsson, 1973) requires the use of agarose plates containing wells for both reactants (see Materials and Methods section). Antibody and antigen are then allowed to diffuse into the gel with the immunoprecipitate being formed at the point of equivalence for each antibody-antigen system. There are many patterns available for cutting wells into the gel, but the most usual is a central well containing one reactant, with eight wells cut in a circle around it at a distance of approximately 1 cm (Ouchterlony, 1962; 1967). For semiquantitative analysis, serial dilutions of the sample are usually placed in the surrounding eight wells, with the relevant antibody in the centre.

The first studies of human red cell membrane proteins were made by Howe et al., 1963. who examined water and butanol extracts of membranes using both double diffusion and immunoelectrophoresis

(q.v.). When double diffusion is used to study integral membrane proteins, a detergent, usually non-ionic, has to be incorporated into the gel in order to keep them in solution. It is necessary to ensure that the presence of even low concentrations (0.1% - 1% v/v) does not introduce artefacts which could lead to mistaken conclusions (see Langdon, 1974; Carey et al., 1975).

(c) Immunoelectrophoresis

The quantitative immunochemical analytical method of choice for membrane protein characterization must be agarose gel immunoelectrophoresis because of the high resolving power, versatility, sensitivity and speed.

Bjerrum and Lundahl (1973; 1974) have demonstrated the use of quantitative immunoelectrophoretic techniques in the analysis of red cell membrane proteins, while other groups have extended it to cover membranes isolated from other sources (Gurd et al., 1973; Bock et al., 1974; Schmidt-Ullrich et al., 1975; Johanssen & Hjerten, 1974; Blomberg & Raftell, 1974; Owen & Salton, 1975; Bhadki et al., 1975; Nielsen & Bjerrum, 1975) such as liver cells, lymphocytes, bacteria, milk fat globules and rat brain synaptosomes.

The technique incorporates the use of specific antisera raised in rabbits against either whole membranes or complex glycoprotein extracts, followed by an electrophoretic sieving in agarose gels on the basis of imparted charge and antigenicity (Bjerrum & Lundahl, 1973; 1974). Several investigators have shown that immunoelectrophoretic analysis of proteins treated with sodium dodecyl sulphate

is possible (Corry & Stone, 1969; Furthmayr & Timpl, 1970; Neeman et al., 1972; Fukui et al., 1971; Smith & Shapiro, 1974), while Bjerrum et al. (1975) have defined conditions that permit quantitative immunoelectrophoresis of detergent solubilized red cell membrane glycoproteins (see also Bjerrum & Bog-Hansen, 1976).

The methods pioneered by Bjerrum & Lundahl and by other Scandinavian workers such as Weeke, Axelson & Kroll are based on the electrophoretic migration of antigens in an antibody containing gel, coupled with a specific immunoprecipitation of these antigens by the corresponding antibodies. Thus, individual immunoprecipitates are formed for each antibody-antigen system that is present. The method is quantifiable because the area enclosed by each precipitate is directly proportional to the antibody:antigen ratio. If the conditions for electrophoresis are carefully chosen, only those antigen molecules with electrophoretic migrations that differ from that of the antibody molecules, will move during electrophoresis. At the start of a run the antigen molecules migrate from the application well into the antibody containing gel. At this stage the number of antigen molecules exceeds the antibody molecules. There follows the formation of small soluble immunocomplexes which will continued to migrate, albeit at a slower rate until more complexes finally fuse to form an immunoprecipitate which is insoluble and does not migrate in the electric field.

The number of antigen and antibody molecules which have combined is termed the "equivalent amount", which was originally defined by Heidelberg and Kendall (1971) but Ingild (1973) suggested that the amount of antibodies giving rise to immunoprecipitates was smaller

than this equivalent amount calculated by the methods of Heidelberg and Kendall. The immunoprecipitate thus formed is relatively unsaturated with antibody, and may be made more distinct by continuing the electrophoresis to saturation. This has been confirmed by Svendsen & Weeke (1967) using slow motion photography. Precipitating antibodies from the sera of many animals have been employed for this technique, but conflicting results have been obtained when horse antisera was used (Clarke & Freeman, 1967; Ressler, 1960; Laurell, 1965). The assay conditions should be such that the antibodies do not move, and this is made possible by performing the electophoresis in agarose gel at pH 8.6, where a balance is achieved between the anodic electrophoretic migration of antibodies and the cathodic backflow caused by electroendosmosis in the high (99%) water content gel. Antigen molecules with mobilities different from the antibodies will form precipitates on the anodic or cathodic side of the application wells. Clarke and Freeman (1967) demonstrated that for the area enclosed by anodic precipitates in crossed immunoelectrophoresis that:

$$\text{Area} = K \times \frac{Ag}{Ab}$$

Thus quantitative measurement of a membrane protein may be made by evaluation of the precipitate area obtained under standardized conditions. For specific procedures see Methods section.

Immunoprecipitates may be detected by staining for protein (amido black or Coomassie Blue), carbohydrate (periodic acid-Schiff) or enzyme activity (Uriel, 1971; Brogren & Bog-Hansen, 1975). The technique can be varied in many ways, with an electrophoresis or isoelectric focusing in the first dimension (Schmidt-Ullrich et al., 1977) with the incorporation of lectins either in this dimension in

order to modify the mobility of a selected component, or in an intermediate gel in the second dimension to modify the shape and extent of the immunoprecipitate.

(iv) Chemical Analysis

The first step requires that the membrane protein/glycoprotein be split into its constituent components. The carbohydrate component may be isolated by exhaustive digestion of the macromolecule with a powerful protease (e.g. trypsin) or a mixture of proteases and glycosylases (pronase). This treatment provides a complex mixture of oligosaccharides with the minimum number of amino-acid residues attached (Spiro, 1973; Sharon, 1975). The resulting glycopeptides may then be fractionated using gel filtration or ion exchange chromatography (Kawasaki & Ashwell, 1976; Harrison et al., 1975; Harrison, 1977; Farrar & Harrison, 1978). As previously described, oligosaccharides free from amino acid residues are released from O-glycosidic by alkaline hydrolysis of the bond between serine or threonine and N-acetylgalactosamine. If alkaline borohydride is used then there is formation of N-acetylgalactosaminitol and 2 amino-acrylic acid (from serine) or 2-aminocrotonic acid (from threnine). These chanages are particularly useful in determining the nature of the carbohydrate-protein linkage (Sharon, 1975; Farrar & Harrison, 1978). Japanese researchers in particular have favoured the release of core oligosaccharides using endoglycosidases (Kohno & Yamashima, 1973; Koide & Muramatsu, 1974; Takasaki & Kobata, 1976).

Amino acid analysis has been performed on several species of

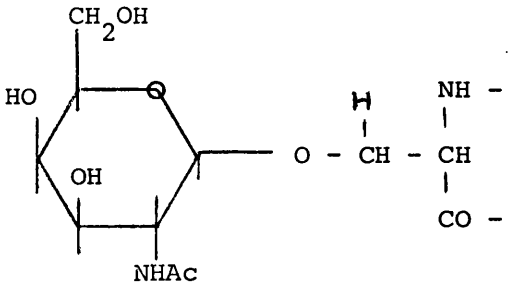
polypeptide (Ozols, 1972; Furthmayr et al., 1975; Hudgin et al., 1974; Farrar & Harrison, 1978). N-terminal analysis has presented no real problems although several membrane proteins have been reported to possess blocked N-termini (Tanner, 1978). A technique developed by Cleveland et al. (1977) involves proteolytic digestion in the presence of SDS, followed by peptide mapping on polyacrylamide gels. Only a limited number of intrinsic membrane glycoproteins have been subjected to complete or partial sequence analysis (Ozols, 1972; Tomita & Marchesi, 1975). For a review on microsequencing see Letarte (1978).

(a) Sugar Analysis

The question of how much sugar a glycoprotein actually contains, although apparently simple to answer, in fact can prove to be quite difficult (Marshall & Neuberger, 1972). Total carbohydrate in glycoproteins may be estimated using colourimetric methods (Dische, 1962; Ashwell, 1966). The phenol-sulphuric acid method for the measurement of total hexose is well established, while other methods are more specific such as the cysteine-sulphuric acid method. Complete quantitative analysis however, requires liberation of the monosaccharide from its glycosidic linkage because, with the exception of the carboxyl groups on sialic acids, the only groups which will react towards chemical analysis without hydrolysis are hydroxyl groups.

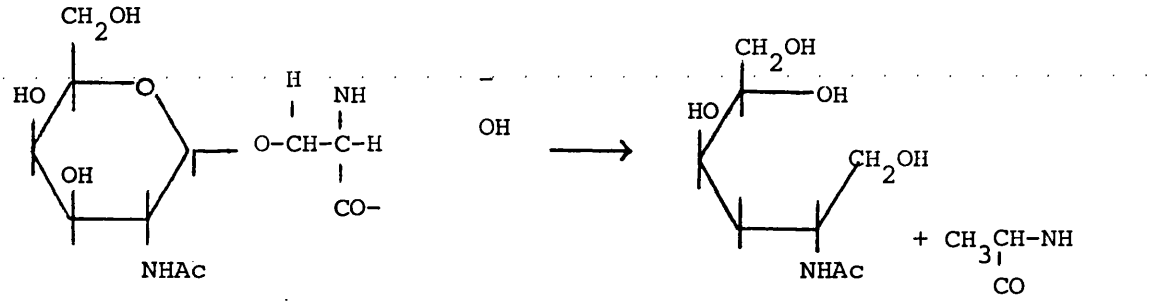
Invariably the method of choice for the liberation of sugars from glycoproteins is acid hydrolysis because only O-glycosidic linkages are susceptible to alkaline hydrolysis via a β-elimination

reaction:



serine

N-acetylgalactosamine



Alanine

N-Acetylgalactosaminitol

Hydrolysis using acid conditions is much more difficult in carbohydrate analysis than it is for amino-acid analysis because monosaccharides generally vary greatly in their stabilities towards hot acid. The hexosamines are more resistant than most, but even so between 5 - 15% of amino sugars such as glucosamine and galactosamine are destroyed on heating in 4 M HCl at 100°C for 16 h. Sialic acids are rapidly destroyed on heating with dilute mineral acids, while those aldoses such as mannose and galactose which do not contain nitrogen occupy something of an intermediate position between the two extremes. The formation of Maillard components by reaction of free sugars with amino acids such as tryptophan, cysteine and methionine, and the reaction of primary or secondary hydroxyls to form oligosaccharides by acid reversion are further complications which may be eliminated by carrying out the hydrolysis at low sugar concentrations.

The acid hydrolysis of most glycosides is thought to proceed via a unimolecular mechanism and to involve a preliminary protonation of the glycosidic oxygen (Figure 4). This protonation, leading to the conversion of compound I to II is followed by a slow breakdown of compound II (the conjugate acid) to the cyclic carbonium ion, which is readily attacked by water to give the free sugar (IV).

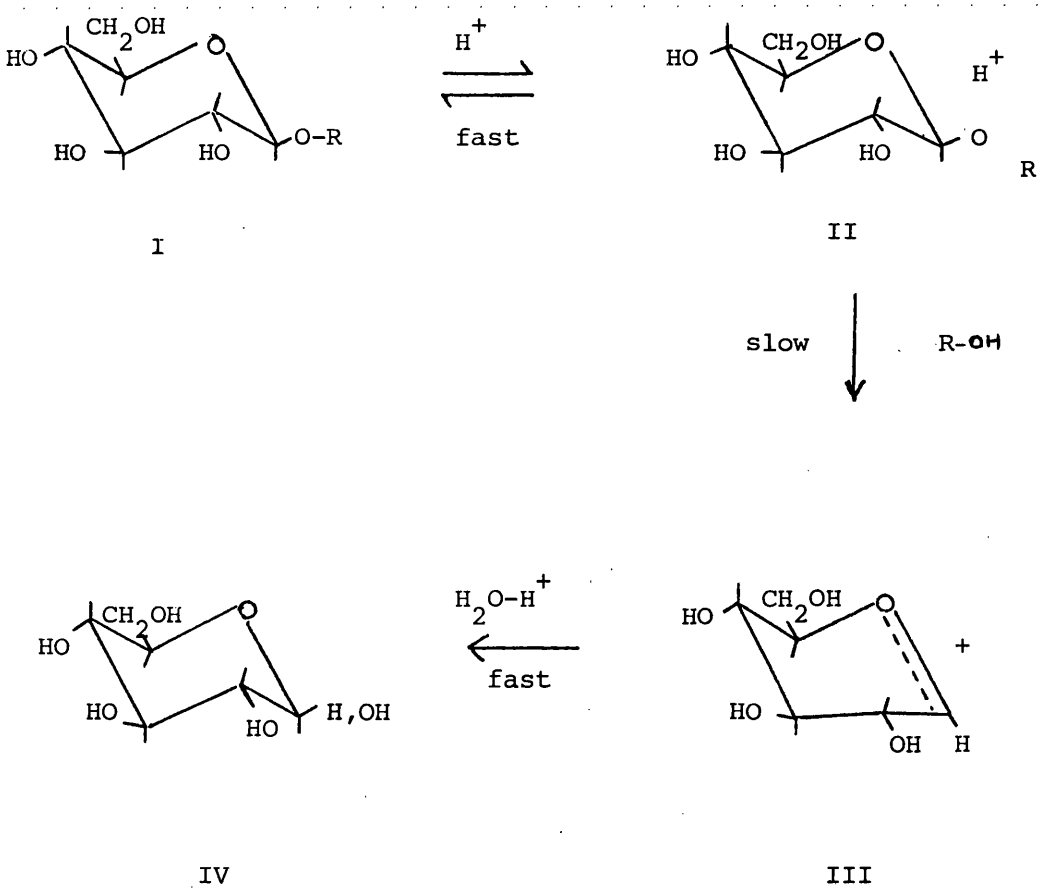


Figure 4. The Mechanism of Acid Hydrolysis of Glycosidases

The overall rate of hydrolysis depends on several factors such as the character of the aglycon (R), the conformation of the molecule and the size of the ring. The degree of protonation too probably plays an important part. Sialic acid residues are very readily removed from glycoproteins, hydrolysis with 0.05 M H_2SO_4 at 80°C for 1 h, being usually sufficient. There are two reasons for this: (i) neuraminic acid resembles other 2-deoxysugars, the glycosides of which are hydrolyzed between 500 - 1000 times more readily than the corresponding glucose derivatives; (ii) a glycoside of N-acylneuraminic acid is a ketoside which might be expected to be hydrolyzed more rapidly than an aldopyranoside. The 6-deoxyhexose, L-fucose is also about five times more readily hydrolyzed that the corresponding galactopyranosides or twenty five times more readily than glucopyranosides. Fucose is easily removed from glycoproteins using mild acid conditions.

(b) Acid hydrolysis of amino sugars

Moggridge and Neuberger (1938) suggested that the great resistance of α or β methylated glucosamine to acid hydrolysis was caused by the presence of a positive charge in close proximity to the glycosidic linkage. They also proposed that methyl N-acetylglucosamine was hydrolyzed along two pathways (Figure 5). The rate constants k_1, k_2 and k_3 were all of the same order of magnitude but the rates of hydrolysis of α or β methylglucosamine (k_4) were about 250 times lower.

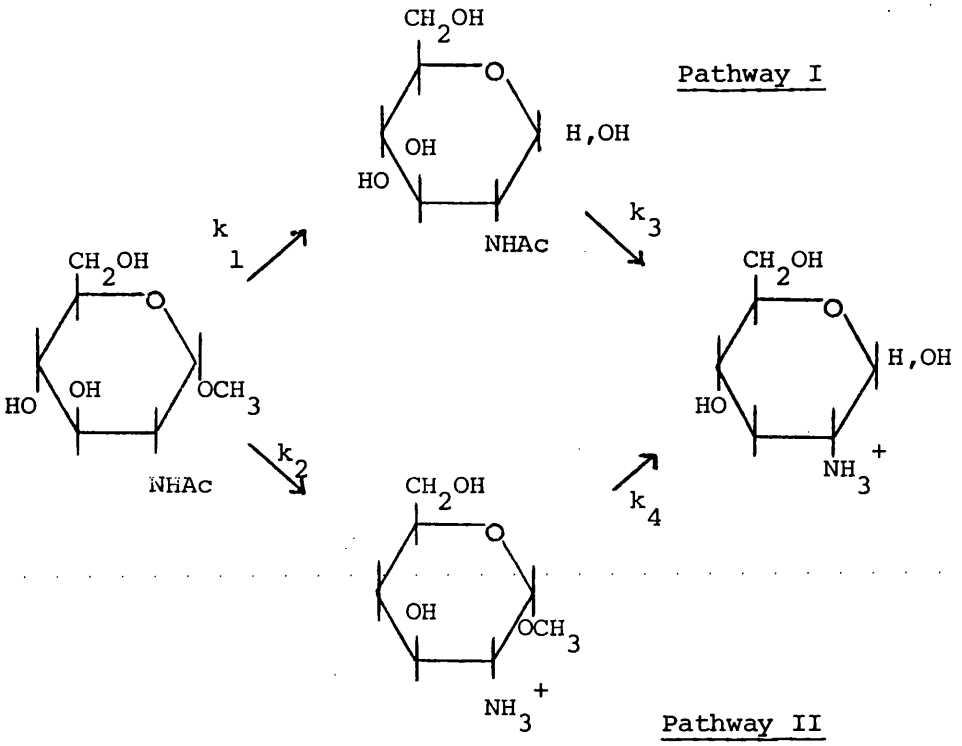


Figure 5. Pathways for Hydrolysis of Methyl N-acetylglucosamine

Since almost all glycoproteins contain N-acetylhexosamine residues it is not too surprising that similar problems are encountered in the hydrolysis of all natural products containing hexosamine residues. Furthermore, any hexose residue to which N-acetylhexosamine is glycosidically linked might be incompletely liberated if hydrolysis is carried out under the conditions usually employed for neutral sugars. Conditions should thus be chosen in order that as large a proportion as possible of the glucosamine present should be liberated by pathway I. It appears that high concentrations of acid (3 M-4M) and temperatures of about 100°C satisfy these requirements. Concentrations of acid and temperatures above this tend to lead to marked destruction of glucosamine. Once the individual monosaccharides have been released by hydrolysis, they may be identified and estimated by a number of procedures, which include paper and thin layer

chromatography, paper electrophoresis and gas-liquid chromatography. This latter technique which requires the sugars to be converted to a volatile derivative, has fast become the technique of choice for both identification and quantitation.

Enzymic assays, the measurement of reducing power (Fehlings solution) and colourimetric techniques are still widely employed. Glucose and galactose oxidase are popular and highly specific enzymes used for sugar analysis.

Another technique which causes less destruction of monosaccharides than acid hydrolysis is methanolysis. The methyl glycosides which result are converted to a volatile derivative to be quantified by gas-liquid chromatography. Relevant articles and reviews are: BeMiller (1967); Spiro (1966); Clamp et al. (1971).

(c) Glycosidases

Enzymic digestion of the glycopeptide is a very useful technique, since it is applicable not only for the isolation of the carbohydrate-peptide linking group, but also for elucidating the structure of the intact glycopeptide. Exoglycosidases, which remove sugar residues singly from the non-reducing end of oligo or polysaccharides, are almost exclusively used (Table 3(i).

Using mannosidase and β-N-acetylglucosaminidase from Jack bean meal, Lis et al.(1969) were able to reduce the soybean agglutinin down to a monosaccharide and single amino acid which comprised the carbohydrate-peptide linking group, the final product being identified

Enzyme	Source
Neuraminidase	Clostridium perfringens
	Diplococcus pneumoniae
	Vibrio cholera
β-N-acetylglucosaminidase	Beef kidney
	Diplococcus pneumoniae
	Jack bean
	Proteus vulgaris
α-Mannosidase	Charonica lampas
	Jack bean
	Proteus vulgaris
β-Mannosidase	Jack bean
	Hen oviduct
β-Galactosidase	Aspergillus niger
	Charonica lampas
	Diplococcus pneumoniae
	Escherichia coli
α-Galactosidase	Coffee beans
	Charonica lampas
α-L-fucosidase	Beef kidney
	Charonica lampas
α-Nacetylgalactosaminidase	Lumbricus terrestris
	Porcine liver

Table 3(i) Glycosidases Available for Structural Studies on Glycoproteins

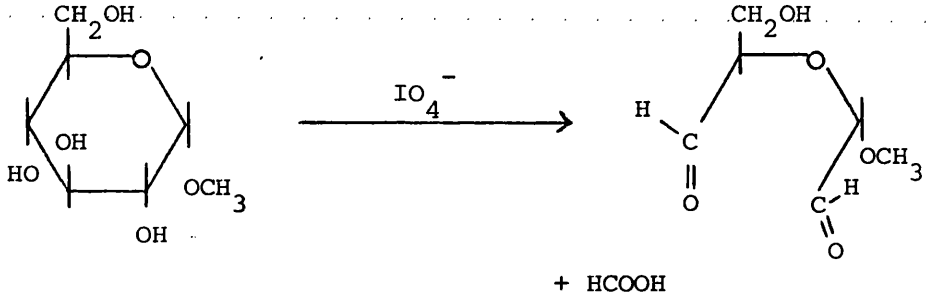
as GlcNAc-Asn, a linkage common to many animal glycoproteins.

Although most glycosidases readily remove sugars from short glycopeptides, they invariably do not remove them so readily from intact glycoproteins. A prime example of the type of enzyme is α-mannosidase. Certain other glycosidases however, will remove sugar residues from intact glycoproteins without any difficulty. Neuraminidase exemplifies this type of enzyme, although of course N-acetylneuraminic acid residues are always found in a terminal position on glycoproteins. An endoglycosidase was isolated from cultures of Streptomyces griseus by Tarentino et al. (1974). This endo-β-N-acetylglucosaminidase H, removes side chains composed of N-acetylglucosamine and mannose from glycopeptides and glycoproteins reduced to their mannose core. The enzyme acts by cleaving the bond between the GlcNAc-Asn and the rest of the carbohydrate side chain.

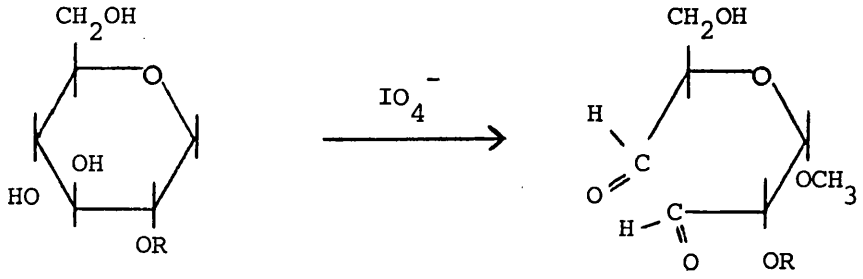
(d) Methylation and Smith degradation

Chemical techniques are also widely used in addition to enzymes for structural studies of the carbohydrate units of glycoproteins. One approach which has been extensively used is the premethylation of free hydroxyl groups, followed by acid hydrolysis and glc analysis of the partially methylated monosaccharides. Hakomori in 1964, developed a procedure that employs methyl iodide and the dimethylsulphinyl carbanion which is a powerful nucleophile. The use of this reagent leads to rapid and complete methylation of all free hydroxyls as well as N-methylation of the acetamide group in hexosamine residues without loss of N-acetyl groups. Such methylation is usually performed on isolated glycopeptides and rarely on intact glycoproteins.

Periodate oxidation, which forms part of the Smith degradation method (Goldstein et al., 1965) is another very useful approach. The procedure which involves periodate oxidation, borohydride reduction and mild acid hydrolysis enables information on the sequencing of sugar residues to be obtained. If methyl α-D-glucopyranoside is taken as an example it will be seen that oxidation with sodium periodate yields a dialdehyde and one mole of formic acid per mole of glycoside. In the process two molecules of periodate are used up.

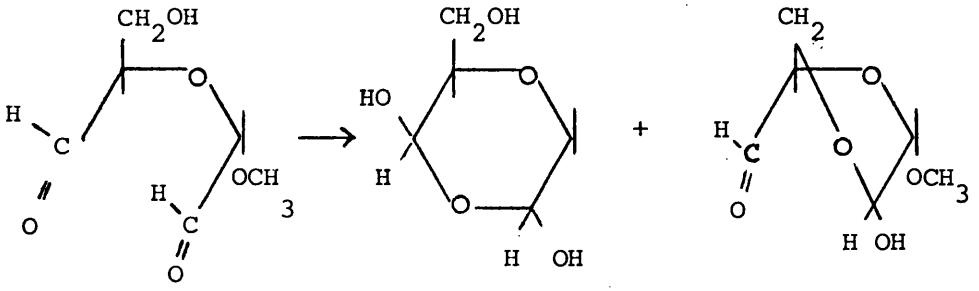


The same result will be obtained if the glycoside is substituted at the 6 position. If however, the substitution is at the 2 or 4-hydroxyl then only one mole of periodate per mole of glycoside is used up. A dialdehyde is formed but no formic acid is produced.

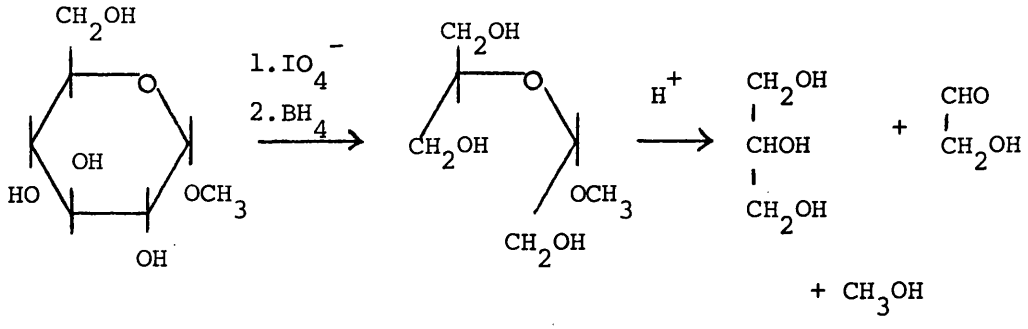


Sugars that are either 3-O-substituted or 2,4-O-disubstituted so that they contain no vicinal diol structures, are resistant to periodate oxidation.

The dialdehydes obtained by periodate oxidation readily form cyclic acetals which are relatively acid-stable:



If however the aldehyde groups are reduced to the corresponding alcohols, the products obtained being true acetals are acid sensitive. The rate of hydrolysis is 10^5 times faster than that of methyl α-glucoside, and it is possible to achieve virtually complete hydrolysis of these acetals without significant effect on glycopyanosidic linkages:



(e) Gas-liquid chromatography

The first account of gas-liquid chromatography (GLC) was published by Martin and James in 1952 and Golay (1957) pioneered the use of coated capillary columns. The method provides a rapid and efficient means of partitioning compounds of similar structure, and when used in conjunction with flame ionization detectors renders possible the analysis of very much smaller quantities of materials than can be

achieved by other techniques. These features were particularly desirable for the determination of the carbohydrate composition of glycoproteins because the large quantities of amino acid present generally interfere to a greater or lesser extent with most colourimetric assays.

Early attempts in the development of this technique used trimethylsilyl derivatives (Sweeley et al., 1963) or methyl glycosides (Clamp et al., 1967) but multiple anomeric peaks were obtained and although this derivatized alditols gave single peaks it was not possible to resolve galactose and mannose or to quantitate amino sugar derivatives. It was later shown by Crowell and Burnett (1967) and Lehnhardt and Winzler (1968) that the alditol acetates of neutral sugars were amenable to glc analysis, and Niedermeyer (1971) extended these observations to include amino sugars.

Gas-liquid chromatography is essentially a method for the rapid and efficient partitioning of compounds which have a similar structure. Volatile derivatives of the compounds to be analyzed are injected into a carrier gas stream which transports them over a column containing a porous solid coated with a thin film of non-volatile liquid (stationary phase). Separation of the components in the gas phase is effected by minute differences in solution behaviour, whereby each component distributes itself between the gas phase and the stationary liquid phase according to their partition coefficients. The porous solid functions only as a support for the liquid stationary phase, enabling it to present a large surface area to the gas phase.

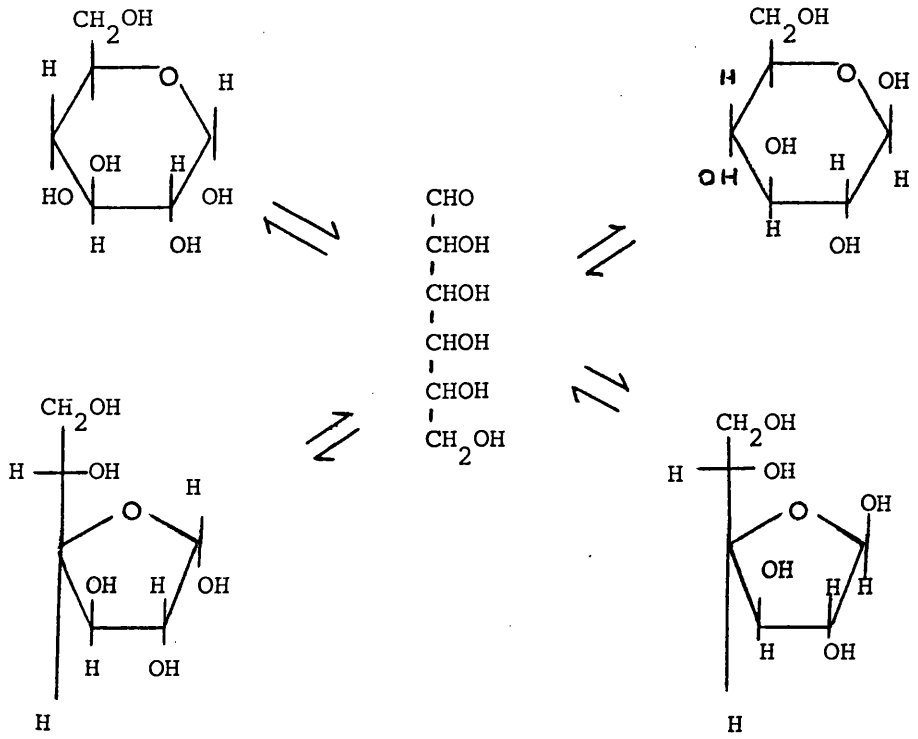
(f) Silyl ethers

There are a number of silyl compounds capable of reacting with the hydroxyl groups of carbohydrates (Pierce, 1968) and Birch (1973) has reviewed their application in the glc analysis of carbohydrates.

$$R(OH)_x + (CH_3)_3SiCl \xrightarrow{\text{Pyridine}} R\ OSi(CH_3)_3{}_x$$

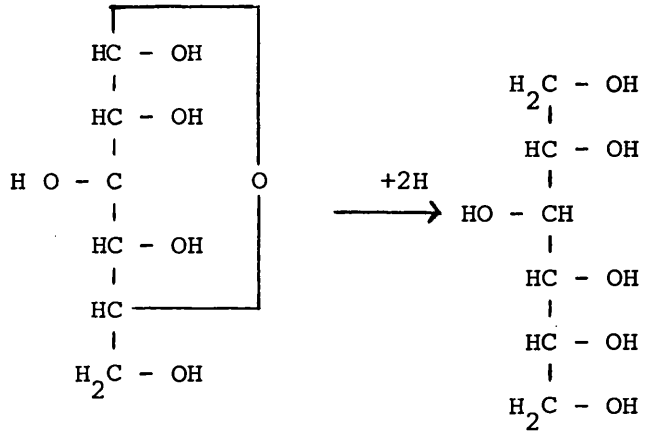
$$+ (CH_3)_3Si\text{-}NH\text{-}Si(CH_3)_3$$

The reaction of a mixture of trimethylchlorosilane and hexamethyl disilazane in pyridine with methyl α glucopyranoside at room temperature, resulted in a 90% yield of methyl (tetra-O-trimethylsilyl)α-glucopyranoside (Sweeley et al., 1964). Any trace of water in these systems may result in mixtures of partially trimethylsilylated derivatives and multiple peaks which can be avoided if the reaction is carried out in a mixture of N,N-dimethylformamide and dimethylsulphoxide (Bentley & Botlock, 1967; Ellis, 1969). It is well known that solutions of some carbohydrates undergo anomerization resulting in an equilibrium mxture through mutarotation:



TMS derivatives are relatively stable to anomerization in comparison to the original sugars. Although four peaks per hexose may be formed, two are usually seen, which correspond to the α and β anomers at the primary carbon atom (Ellis, 1969).

One approach which reduces the number of anomeric peaks seen in the chromatogram requires that individual monosaccharides are reduced to their corresponding alditols. These are then converted to their TMS, or more usually to their acetate derivatives which tend to give a better resolution.



D-glucose Sorbitol

(g) Methylation

Fully methylated sugars have a high volatility and even oligosaccharides have been separated in this way (Karkkainen, 1971). The Hakomori method is most often used (see above).

$$R(OH)_x + NaH \xrightarrow[CH_3SOCH_2^-]{CH_3SOCH_3} R(ONa) \xrightarrow{CH_3I} ROCH_3 + NaI$$

(Hakomori, 1964).

For reviews on glc see Dutton (1973; 1974); Bishop (1962; 1964); Kircher (1962); Wells et al. (1964); Clamp et al. (1971).

1. MATERIALS

1.1 Electric Fish

Deep frozen electric organs from Torpedo marmorata were supplied by the Marine Institute at Arcachon. They were stored at -20°C until required.

1.2 Chemicals and Enzymes

All solutions used were prepared with glass-distilled water. General chemicals were obtained from BDH Ltd., Poole, Dorset, and were of Analar grade unless otherwise stated. Pronase (B grade) from Streptomyces griseus was obtained from Calbiochem Ltd. (London) and monosaccharide sugar standards from Sigma Ltd., Surrey. Protein standards for polyacrylamide gel electrophoresis were purchased from Boehringer Ltd., London. Silyl 8 and Trisyl are products of the Pierce Chemical Co., and were obtained from Pierce-Warriner (Chester). Both reagents contain hexamethyldisilazane and trimethylchlorosilane in dry pyridine. Liquid phase and solid support media were purchased from Phase Separations Ltd., Clwyd.

1.3 Apparatus

For gas-liquid chromatographic analyses, Perkin-Elmer F11 and Pye Unicam series 104 gas chromatographs were used, both of which were equipped with a flame ionization detector system. Immunoelectrophoresis was performed using an LKB Multiphor System. For disc polyacrylamide gel electrophoresis a Shandon-Southern apparatus was employed, while polyacrylamide gradient gel electrophoresis was performed with a system obtained from Uniscil Ltd., London.

1.4 Glassware

All glassware used in sugar determinations and analyses was cleaned by immersion in alkaline permanganate solution (1% w/v) followed by a wash in hydrochloric acid (2M) and rinsed in glass double-distilled water.

2. METHODS

2.1 Equilibration of Sephadex Gels

The gels used were swollen in double distilled water containing 0.02% NaN_3 at 4°C according to the procedures detailed in the booklet "Sephadex-Gel filtration in Theory and Practice" (Pharmacia Fine Chemicals AB; Uppsala, Sweden).

2.2 Immunization of Rabbits

Acetylcholine receptor-rich membrane fragments were prepared as detailed in the Results section, and aliquots (1 ml containing approximately 2.5 mg protein) were emulsified in Freund's complete adjuvant (Calbiochem Ltd.) (10 ml). Male New Zealand white rabbits were immunized (0.5 ml, subcutaneously) at weekly intervals for 4 weeks, and subsequently at 5-weekly intervals. Samples (50 ml) of blood were collected by arterial puncture every 6 weeks, commencing 10 weeks after the first immunization, and serum prepared by allowing the blood to clot.

2.3 Preparation of Rabbit Immune IgG Fraction

Saturated aqueous ammonium sulphate solution was added dropwise

to serum (50 ml) with continuous stirring at 0^{o}-4^{o}C. The mixture was allowed to stand for 15 min. and centrifuged (2,000 x g, 10 min). The pellet was washed twice with saturated ammonium sulphate solution (50 ml) centrifuged as before and the supernatant discarded. The resulting pellet was dissolved in 0.9% NaCl solution (50 ml) and the globulin fraction was re-precipitated using saturated ammonium sulphate solution (25 ml), washed twice with the same solution and dissolved in distilled water (5 ml), before being desalted by elution from a Sephadex G-25 column. The eluate was monitored at 280 nm and the recorder trace showd a three peak pattern (Figure 6). Fractions corresponding to each peak were pooled, freeze-dried and re-dissolved in distilled water followed by dialysis overnight at 4^{o}C against double distilled water. The resulting non-diffusable samples were analysed by immunodiffusion against AChR-rich membrane fragments solubilized in Triton X-100 (q.v.) in agarose gel (Figure 7).

The pool from fractions 10 through 18 gave the only precipitin line in this assay. Aprotinin (Sigma) was added (500 IU per ml) and the solution stored frozen in aliquots of 200 µl until required for assay. The active peak corresponded with the position of immunoglobulin fraction G (IgG).

2.4 Preparation of Antigen Solution

AChR-rich membrane fragments (approximately 25 mg protein) were suspended in 20 ml of Triton X-100 solution (1% v/v) and stirred at 5^{o}C for 2h. followed by centrifugation (100,000 x g) for 1 h. The pellet thus obtained was treated in the same fashion and the two supernatants pooled, freeze-dried and resuspended in physiological saline (5 ml).

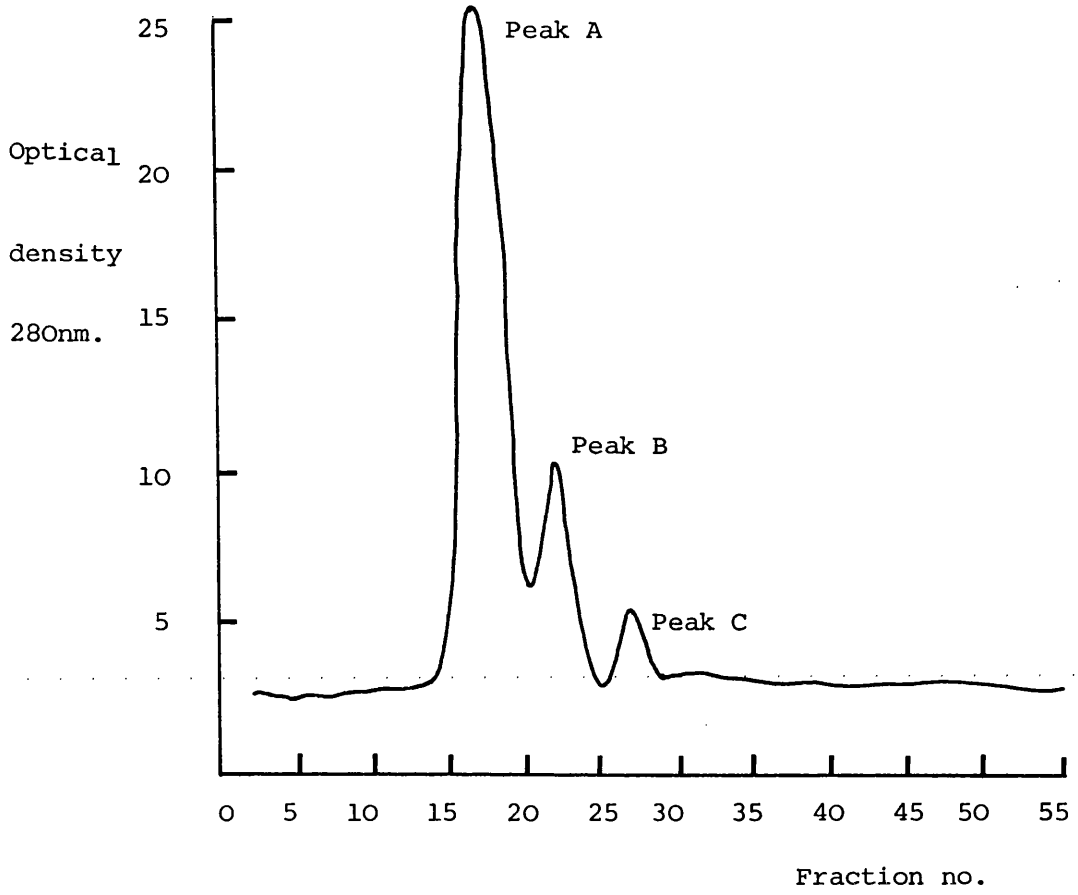
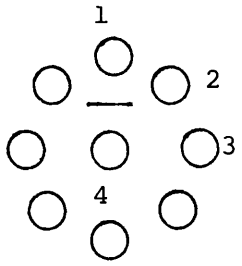


Figure 6. Preparation of rabbit immune Ig fraction: Elution pattern of serum treated with saturated $(NH_4)_2SO_4$ solution from Sephadex G-25. Peak A (fractions 10-18) gave a precipitin line with rabbit immune IgG.

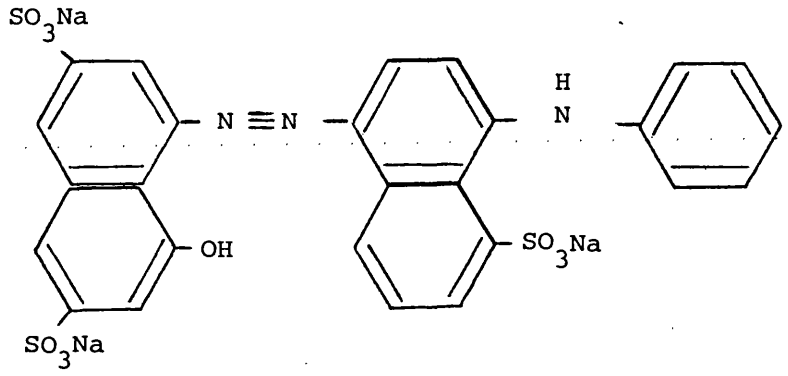


1. = fractions 10-18 (Peak A)
2 = fractions 19-26 (Peak B)
3 = fractions 27-34 (Peak C)
4 = AChR rich membrane fragments dissolved in Triton X-100 (1.% v/v)

Figure 7. Rabbit immune Ig fraction: immunodiffusion assay of eluate from Sephadex G-25 with Torpedo electroplax membrane fragments dissolved in Triton X-100 (1%, v/v).

2.5 Immunoelectrophoresis

The buffer used in all the experiments performed was sodium barbital pH 8.6, I = 0.1. The staining solution contained Coomassie Brilliant Blue R250 (Sigma) (10 g) in 96% ethanol (900 ml), glacial acetic acid (200 ml) and distilled water (900 ml). The destaining solution used contained the above solvents only.



Coomassie Blue

Coomassie Blue has the structure represented above, and in view of its ability to bind to mixed-bed ion exchange resins, probably forms ionic attachments to proteins.

(a) Gel casting

A clean, dry glass plate (8.4 x 9.4 x 0.1 cm) was pre-heated to 90°C and placed on a levelled, horizontal table checked with the aid of a spirit level. The agarose solution (15 ml; 1% in barbital buffer containing 1% Triton X-100) was heated to 55°C and poured onto the plate using a pre-heated measuring cylinder. For rocket immunoelectrophoresis rabbit anti (*Torpedo* membrane) IgG (2% v/v) was added before gel casting. When the agarose had congealed (approximately 10 min.), wells (0.5 cm diameter) were punched

out using a gel punch and perspex template.

(b) Rocket immunoelectrophoresis

The agarose-coated glass plate was placed on the cooling plate in the Multiphor unit and connected to the buffer solution in both troughs via wicks made from 5 layers of Whatman number 1 filter paper (9.5 x 12 cm). To each well was added antigen solution (10 ml), water cooling was started and the power supply immediately switched on in order to avoid the formation of ring precipitates around each well. Ideally, the samples should have been applied with the power switched on. Electrophoresis was performed for either 1h. at 10 V per cm; or 16h at 2 V per cm.

(c) Crossed immunoelectrophoresis

After pouring the plate and the congellation of the agarose, a single well was punched in the lower left hand corner of the plate, 2cm distant from the bottom and left hand side (Figure 8). The plate was placed in the Multiphor unit as detailed above and antigen samples (10 µl) applied. Electrophoresis was carried out for 1 h. at 10V per cm. All of the gel above the well was then removed and agarose (12 ml) containing anti-(*Torpedo* membrane) IgG (2%,v/v) poured in its place. Electrophoresis was then continued in the second dimension (Figure 9) at 2V per cm. for 16h.

(d) Washing, pressing and staining of gels

Non-precipitated proteins were removed after electrophoresis by

washing in 0.9% NaCl solution, followed by double-distilled water. In order to facilitate staining, the gel was pressed to a thin film as follows: (i) the plate was placed on a flat surface and five thicknesses of Whatman number 1 filter paper circles placed on top. (ii) A glass plate (20 x 20 cm) was placed over the filter paper and a heavy weight (usually five or six large books) placed on the plate. (iii) After 30 min the weight, glass plate and filter papers were removed and the proteins heat-fixed by heating the plate in an oven for 15 min. at 100°C. Staining was performed by immersing the plate in the staining solution contained in an enamel dish. The timing was found not to be critical, but at least 10 min. was allowed. The plate was then placed in the de-stain solution until a clear background to the stained precipitates was obtained.

2.6 Polyacrylamide Gradient Gel Electrophoresis (PAGGE)

This was carried out using pre-cast "Gradipore" gradient gels purchased from Uniscil Ltd. Gradipore gels (75 x 75 x 2.8 mm) feature a continuous concave polyacrylamide gradient from 2.5% to 28%, superimposed onto which is a cross-linked gradient increasing from 2.5% to 6.2% relative to monomer.

Samples to be examined were placed in slots in a sample applicator which itself slotted into the top (i.e. low polyacrylamide concentration) of each gel cassette. The cassette was then held by a rubber gasket in contact with both buffer solution chambers each of which contained a platinum wire electrode. Electrophoresis was performed in 0.2 M sodium phosphate buffer, pH 7,2, containing sodium dodecyl sulphate (2%, w/v). Gels were stained by removing

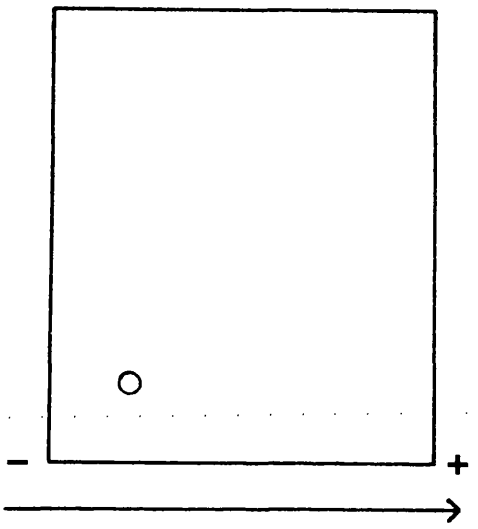


Figure 8. Crossed immunoelectrophoresis: 1st dimension run using 10 µl antigen solution. Electrophoresis performed for 1h. at 10 v per cm.

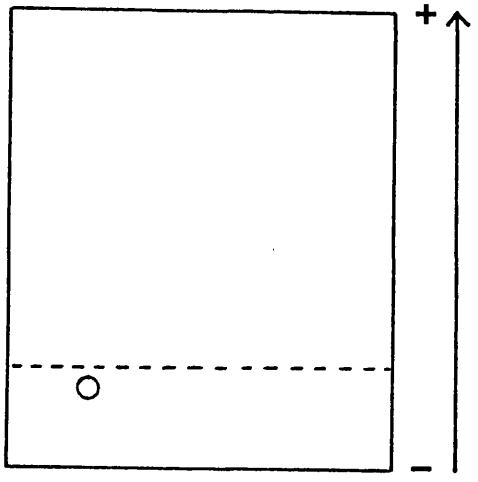


Figure 9. Crossed immunoelectrophoresis: 2nd dimension run. Agarose contained anti (Torpedo membrane) IgG (2%, v/v). Electrophoresis perforomed for 16h. at 2V per cm.

them from the cassette, fixing in 25% isopropanol: 10% acetic acid and immersion in the Coomassie Blue staining solution described above. Destaining was carried out by immersion in fixing solution. The incorporation of glycerol (1% v/v) into the fixing solution helped to reduce gel shrinkage.

2.7 Disc Gel Electrophoresis

The preparation of samples, acrylamide solutions and buffers were according to Weber and Osborn (1969).The stacking gels used had the following composition:-

Solution 1 (pH 6.7)	1 M HCl (48 ml)
	Tris (5.98 g)
	TEMED (0.46% v/v in water)
Solution 2	riboflavin (4%, w/v in water)
Solution 3	sucrose (40%, w/v in water)
Solution 4	acrylamide (10 g)
	methylene-bis-acrylamide (2.5%,w/v in water).

Gels were prepared using the following quantities of each solution:-

Solution 1 (0.5 ml)

Solution 2 (0.5 ml)

Solution 3 (2.0 ml)

Solution 4 (1.0 ml)

Running gels were prepared by dissolving acrylamide (22.2g) and methylene-bis-acrylamide (0.6 g) in water (100 ml). This gave a 10% solution which could be used neat or diluted to give the required acrylamide concentration. The solution was stored in a dark bottle at $4^{o}C$.

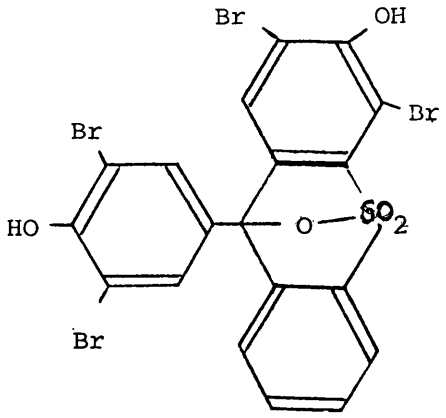
Low concentration stacking gels were employed to "tighten up" the protein bands before migration on the running gels. Some anomalies have been discovered in the use of this technique however.

(i) Procedure

A Shandon-Southern apparatus was used which held eight pyrex tubes (7.5 cm long) open at either end. The bottom of each tube was capped with a "Subaseal" and the running gel (1.2 ml) was pipetted in, the top being overlaid with gel buffer. After polymerization was complete (10 min), the stacking gel solution (0.2 ml) was added by pipette and the top was overlaid with distilled water. Photopolymerization was carried out by placing the gel under a tungsten lamp for at least 30 min. Buffer solution was then added to both chambers and the samples were applied to the tops of the gels under the buffer surface. Electrophoresis was carried out for 3h. at 8 mA per gel using a Vokam powerpack. At completion the gels were removed from the tubes by air pressure from a plastic syringe and immersed in fixing solution for 1h. Each gel was then stained for at least 2½ h. in Coomassie Blue solution, and destained by heating (boiling water bath) in a number of changes of solution containing aqueous methanol (5% v/v) and aqueous acetic acid (7%, v/v).

(ii) Evaluation

Each sample contained Bromophenol Blue as a tracker dye:-



Bromophenol Blue - molecular weight 670 daltons

At the completion of each run, the position of the tracker dye was noted. The distance migrated by each protein component was measured after staining, and the following formula applied to calculate the"relative mobility" (M_r):-

$$M_r = \frac{\text{distance travelled by protein x 100}}{\text{distance of dye migration}}$$

A correction factor may be incorporated into this formula to allow for the shrinkage and swelling of the gels during the staining procedures, but in practice this invariably opproximates to unity.

The relative mobility of each protein component was noted, and the approximate molecular weight was found by reference to a standard curve (Figure 10) of M_r vs. mol. wt. $\log_{10}$. Densitometer tracings were also obtained for each gel using a modified Pye Unicam SP1800 system.

(iii) Staining for carbohydrate

Gels were stained for carbohydrate using the periodic acid - Schiff (PAS) method (Cullen *et al.*, 1974; 1976)

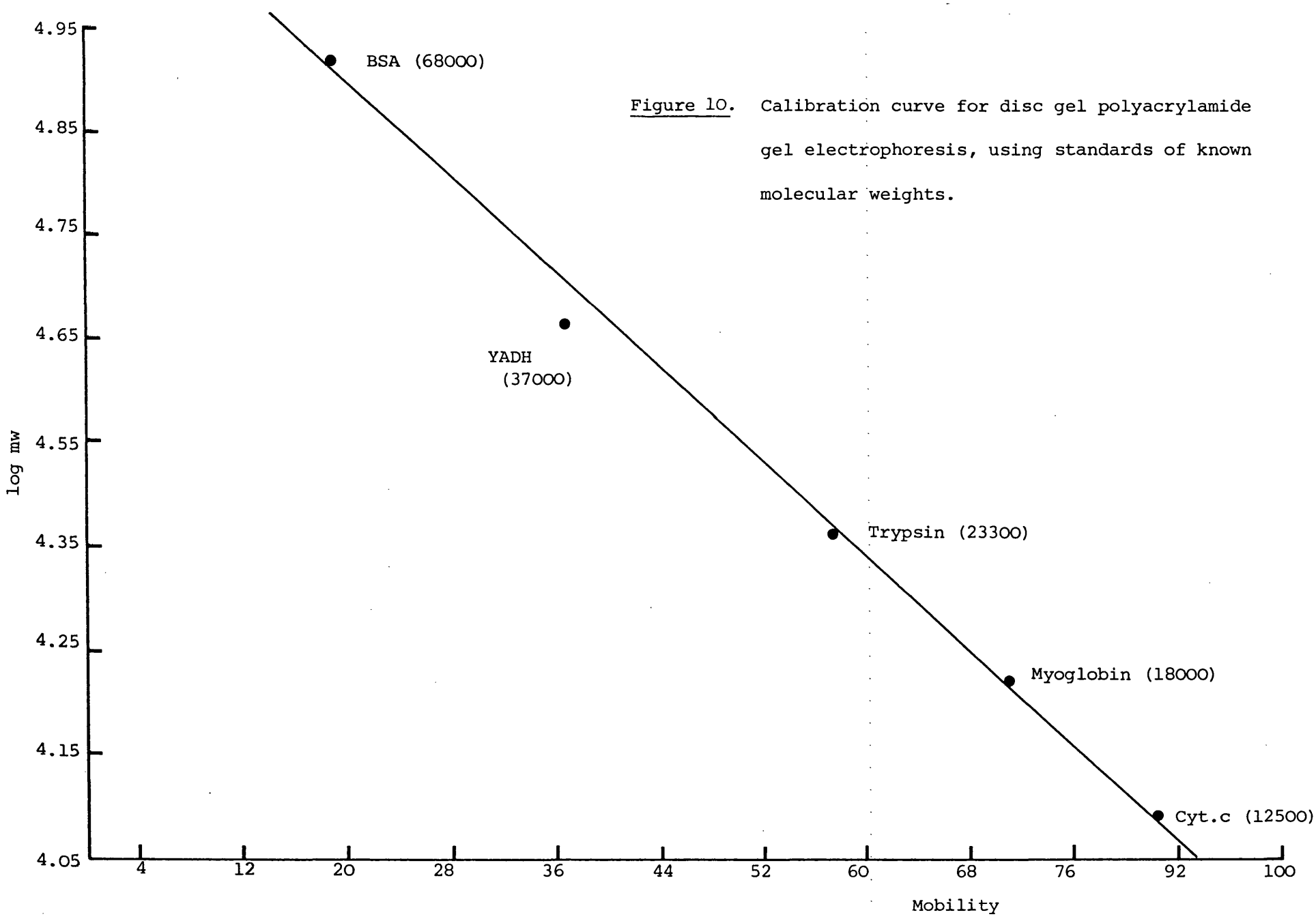


Figure 10. Calibration curve for disc gel polyacrylamide gel electrophoresis, using standards of known molecular weights.

(a) gels fixed in trichloracetic acid (12.5%, v/v) for 1h.

(b) washed in distilled water (15 sec.)

(c) immersed in periodic acid (1% v/v) in acetic acid (3% v/v) for 1h.

(d) immersed in sodium arsenite (0.5% w/v) in acetic acid (5%, v/v) for 30 min.

(e) immersed in acetic acid (5%, v/v), until gel was clear.

(f) immersed in Schiff reagent until a pink colouration was obtained.

3. ANALYTICAL METHODS

3.1 Protein

Three methods were employed:

(a) a modification of the Lowry method

(b) ninhydrin reagent

(c) ultraviolet light absorbance.

(a) The modified Lowry technique

Reagent A - sodium tartrate (1% w/v); copper sulphate (0.5%, w/v) + 1N NaOH

Reagent B - sodium carbonate solution (2%, w/v)

Reagent C - Folin-Ciocalteau solution, diluted 1:1 with distilled water.

Standard protein solution - bovine serum albumin (0.8 g per ml) in water.

Aliquots of the standard protein solution were taken as follows:

Volume	protein concentration
10 µl	8 µg
25 µl	20 µg
50 µl	40 µg
75 µl	60 µg
100 µl	100 µg

and diluted (to 200 µl) with 0.1 N NaOH. To reagent B (50 ml) was added reagent A (1 ml) and an aliquot (1 ml) added to each assay tube. After mixing, the samples were allowed to stand at room temperature for 20 min. and then reagent C (0.1 ml) was added, the contents of each tube mixed and allowed to stand for at least 30 min. Each assay tube was well mixed before measuring the absorbance at 750 nm of each sample. From such measurements a calibration curve of optical density at 750 nm vs. protein concentration was constructed. A reagent blank was used which contained 0.05 N NaOH made up to the correct volume for the concentration required to fit the calibration curve (see Lowry et al., 1951).

(b) Ninhydrin assay

Performed according to Lee and Takahashi (1966).

(c) Ultraviolet light absorption

Two procedures were employed:-

(i) The method of Warburg and Christian (1940)

The absorbance of a suitably diluted protein solution was measured at both 260 nm and 280 nm against a diluent blank.

The following formula was then applied to calculate the protein concentration in mg. per ml.

$$O.D._{280nm} \times 1.55 - O.D_{260nm} \times 0.76 = \text{mg per ml protein}$$

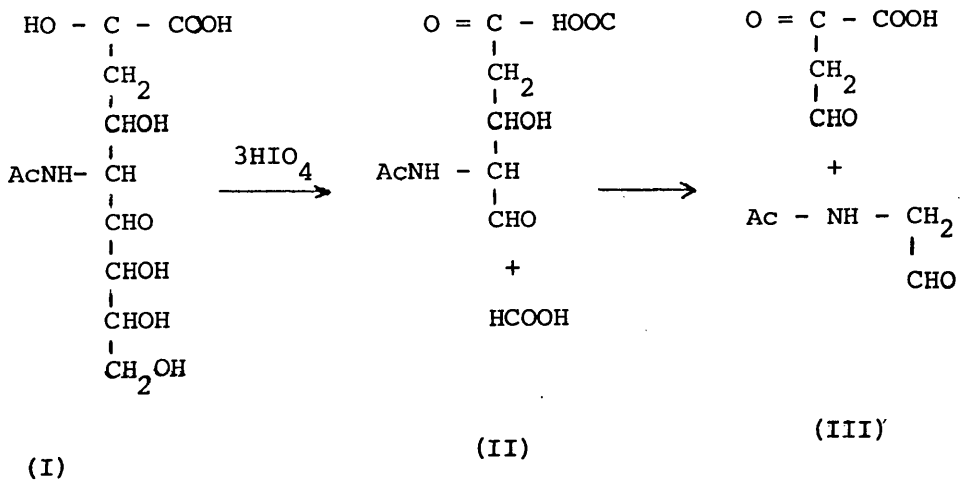
(ii) The Waddel Method (215/225 nm) (Waddel, 1956)

The protein sample was diluted with 0.5M NaCl and the absorbance measured at 215 nm and 225 nm against a diluent blank. The following formula was then applied to give an answer in µg protein per ml.

$$(OD_{215nm} - OD_{225nm}) \times 144 = \mu g \text{ protein per ml}$$

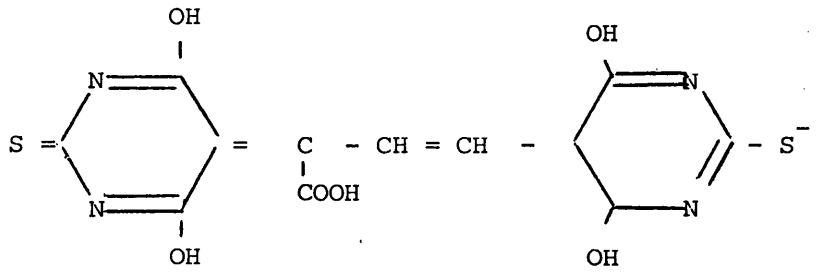
3.2 Sialic Acid

Pronase digest supernatants and Sephadex G-50 column eluates (see results section) were assayed for sialic acid by the Aminoff (1961) modification of Warren's (1959) thiobarbituric acid assay. Periodate cleavage of the sialic acid molecule at bonds between C-8 and C-9, and also C-7 and C-8 yields the chromogen β-formyl pyruvate (III) via the intermediate (II) 2-acetamide-4-deoxy hexos-5 uluronic



(I) (II) (III)

acid. Addition of thiobarbiturate gives the chromophore:-

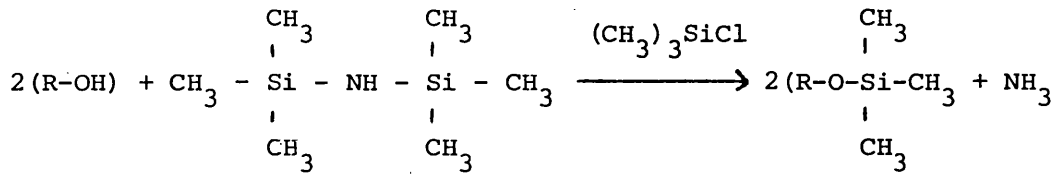


(IV)

3.3 Hexoses and hexosamines

Total hexose was determined colourimetrically by a modification of the cysteine: H_2SO_4 assay of Dische and Danilchanko (1967).

For gas-liquid chromatographic analysis of individual hexoses and hexosamines in soluble carbohydrate containing fractions, aliquots (400 µl) were diluted with an equal volume of 1.2 M HCl, saturated with N_2, sealed in an ampoule and heated at 100°C for 16h. The cooled hydrolysate was freed of HCl either by azeotropic distillation with ethanol: benzene (4:1v/v) for trimethylsilyl ether derivatives, or by repeated evaporation at 80°C under N_2 and addition of 0.1µN NaOH (100 µl) for alditol acetate derivatives. For conversion to their relevant trimethylsilyl ethers, the samples were evaporated to dryness in a stream of N_2. "Trisyl" (50 µl) was added and the mixture was incubated at 37°C for 15 min. Excess reagent was evaporated off and the dry residue was reconstituted in n-hexane. Aliquots of this solution were then chromatographed.

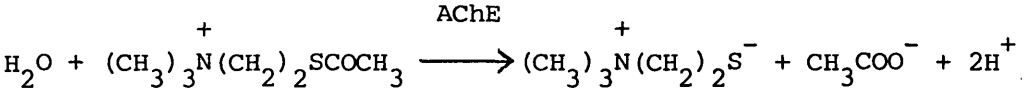


SE 30 chromasorb (80 - 100 mesh) 3%, was used to pack the column which was preconditioned with silyl-8. The running temperature was 195°C.

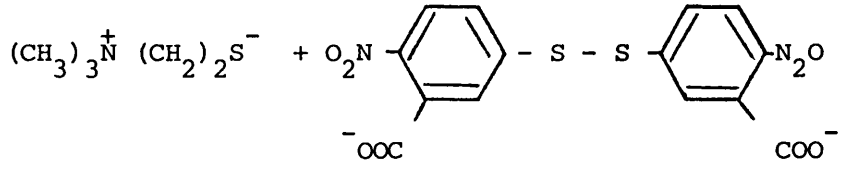
In order to convert sugars to their alditol acetate form, the acid-free sample residue was diluted (to 800 µl) with glass double-distilled water, and 0.1 N NaOH (100 µl) and KBH_4 (2.5% in 0.1 N NaOH - 100 µl) were added. After incubation at room temperature for at least 1h., the borohydride was destroyed by the dropwise addition of 4N acetic acid (200 µl). Excess borate was removed as the volatile tetramethyl ester by repeated addition and evaporation of methanol at 80°C, in a stream of N_2. To the dry residue thus obtained, was added pyridine (500 µl) and acetic anhydride (500 µl) and the sample was incubated at 100°C for 1 h. Excess reagents were evaporated off at 80°C in a stream of N_2 and the residue was taken up in dry, redistilled chloroform. Aliquots of the chloroform solution were chromatographed on a column packed with 3% OV 225 chromasorb W-HP (80 - 100 mesh) at 205°C.

3.4 Acetylcholinesterase (AChE)

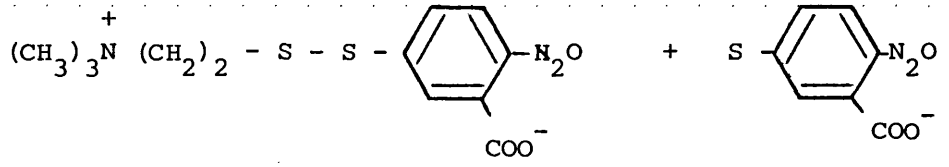
The enzyme was assayed according to the method of Ellman et al. (1961), in which acetylthiocholine (I) is hydrolysed by AChE to give thiocholine (II) which reacts with 5:5 dithiobis-2-nitrobenzoate (III) to give the yellow anion 5 thio-2-nitrobenzoate (IV) and choline dithiobis-2-nitrobenzoate (V).



(I) (II)



(III)



(V) (IV)

The extinction coefficient of IV is 1.36×10^4 moles per l, and so conversion from absorbance units to absolute units was achieved by applying the formula:

$$\frac{\Delta \text{ absorbance per min}}{1.36 \times 10^4} \times \frac{\text{cuvette volume (ml)}}{\text{sample volume (ml)}} \times \frac{10^3}{\text{protein concentration (µg per ml)}}$$

thus enzyme activity was expressed as µmoles of acetylthiocholine hydrolyzed per µg protein per ml.

3.5 Toxin Binding Assay

The stock solution of α bungarotoxin contained 20 Ci per mMole, and the concentration was 2.2×10^{-5} M. This stock toxin solution was diluted 1:10,000 using eel Ringer[1] (1.5 mM sodium phosphate buffer,

1 Meunier, et al., (1974)

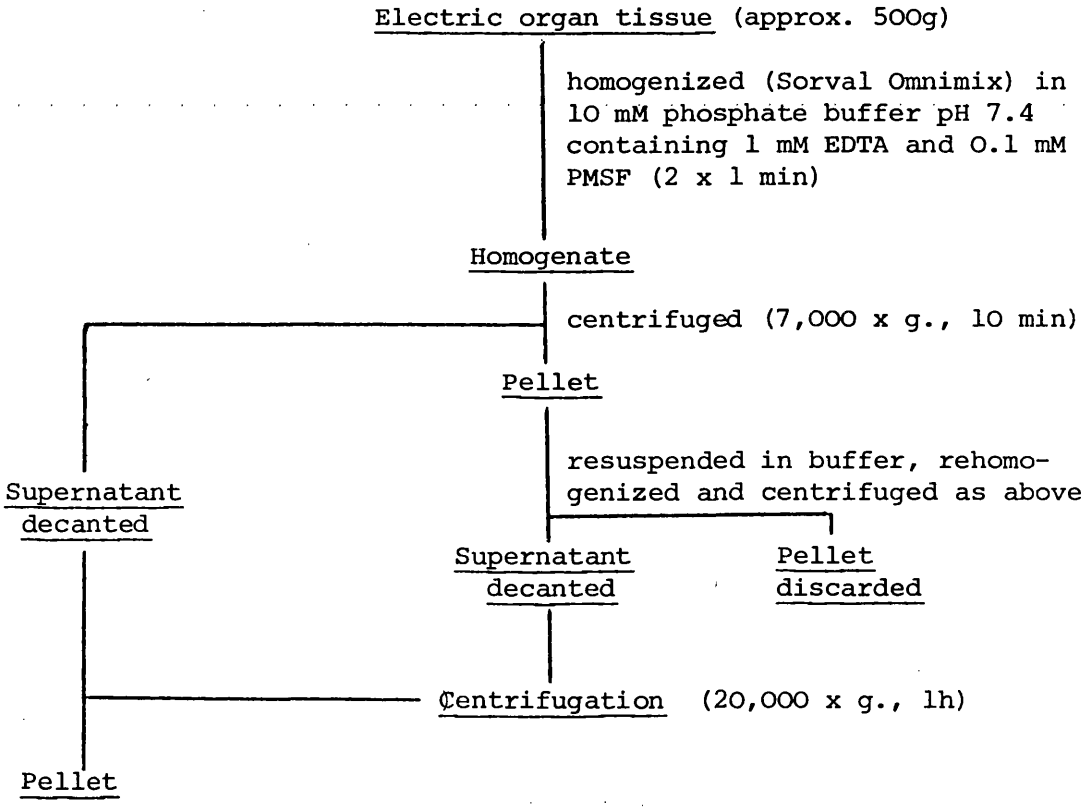
pH 7; 160 mM NaCl; 5 mM KCl; 2 mM $MgCl_2$ and 2 mM $CaCl_2$). Diluted toxin (200 µl) was added to each of a series of tubes together with a sample of membrane preparation, diluted 1:100 with "helper".[1] The range of samples were 1 - 10 µl. Each sample was then incubated for 1h. at room temperature, and diluted to 10 ml with Veel Ringer The samples were filtered through a Millipore HAWP 02500 0.45 µ disc membrane filter and washed with a further 10 ml. Ringers solution. The filter discs were dried for ½ - 1 h. and counted by immersion in 5 ml toluene phosphor (5 g PPO in 1 litre toluene) 2,5 diphenyloxazole (PPO) has a λ_{max} of 365 nm.

[1] The purified receptor protein is not quantitatively retained on Millipore filters under the assay conditions used for crude extracts. Thus the receptor-rich membranes were first diluted with the crude protein fraction which did not absorb to an affinity column, (helper) and was thus depleted of receptor molecules. Helper was always included in the blanks to prevent the excessive binding of toxin to the filters which occurs in the absence of detergent. (Meunier, et al., 1974).

RESULTS

1. PREPARATION OF AChR-RICH MEMBRANE FRAGMENTS FROM THE ELECTRIC TISSUE OF TORPEDO MARMORATA

Membrane fragments, rich in acetylcholine receptor as determined by toxin-binding assay (see Materials and Methods Section p. 83), were prepared from the electric organs of Torpedo marmorata as described by Sobel et al. (1977), according to the following scheme:-



The pellet thus obtained was resuspended in buffer, washed by centrifugation and then stored in buffer containing NaN_3 (0.02%) at $4^{o}C$ until required.

2. PROTEOLYTIC DIGESTION OF MEMBRANE FRAGMENTS

2.1 Digestion with Soluble Pronase (Extract I)

An aliquot of the washed pellet (10 mg protein, determined by Lowry assay) was suspended in 50 mM tris-HCl buffer containing 4 mM $CaCl_2$ pH 7.8 and pronase (1 mg).This mixture was incubated for 30 min at 37°C and the resulting digest mixture was centrifuged (100,000 x g. 1h), the supernatant being retained.

A preliminary experiment had shown that when membrane fragments were incubated with pronase (1 mg) at 37°C, for 24 h., over the pH range 7.4 to 8.2, a maximum amount of material absorbing at 225 nm was released at pH 7.8. It is worth noting however, that when the digests were assayed by ninhydrin (Lee and Takahashi, 1966) the maximal enzymic activity was apparently obtained at pH 7.6 (Figurell). A comparison of the effects of membrane protein:pronase ratios of 50:1 and 10:1 by weight, was performed by suspending 50 mg and 10 mg respectively of membrane protein (assayed by modified Lowry technique) in 10 ml tris HCl buffer (pH 7.8) containing 4 mM $CaCl_2$. The samples were incubated at 37°C for 24 h, centrifuged (30,000 rev. per min for 1h) and the supernatants decanted, freeze dried and weighed. It as found that the 50:1 ratio averaged a release of 19.3% (expressed as a percentage of the original weight of membrane material) while the 10:1 ratio averaged 85.3%. Parallel determinations showed that during digestion, most of the protein was released in the first hour of incubation, with only a small increase thereafter (Table 4).

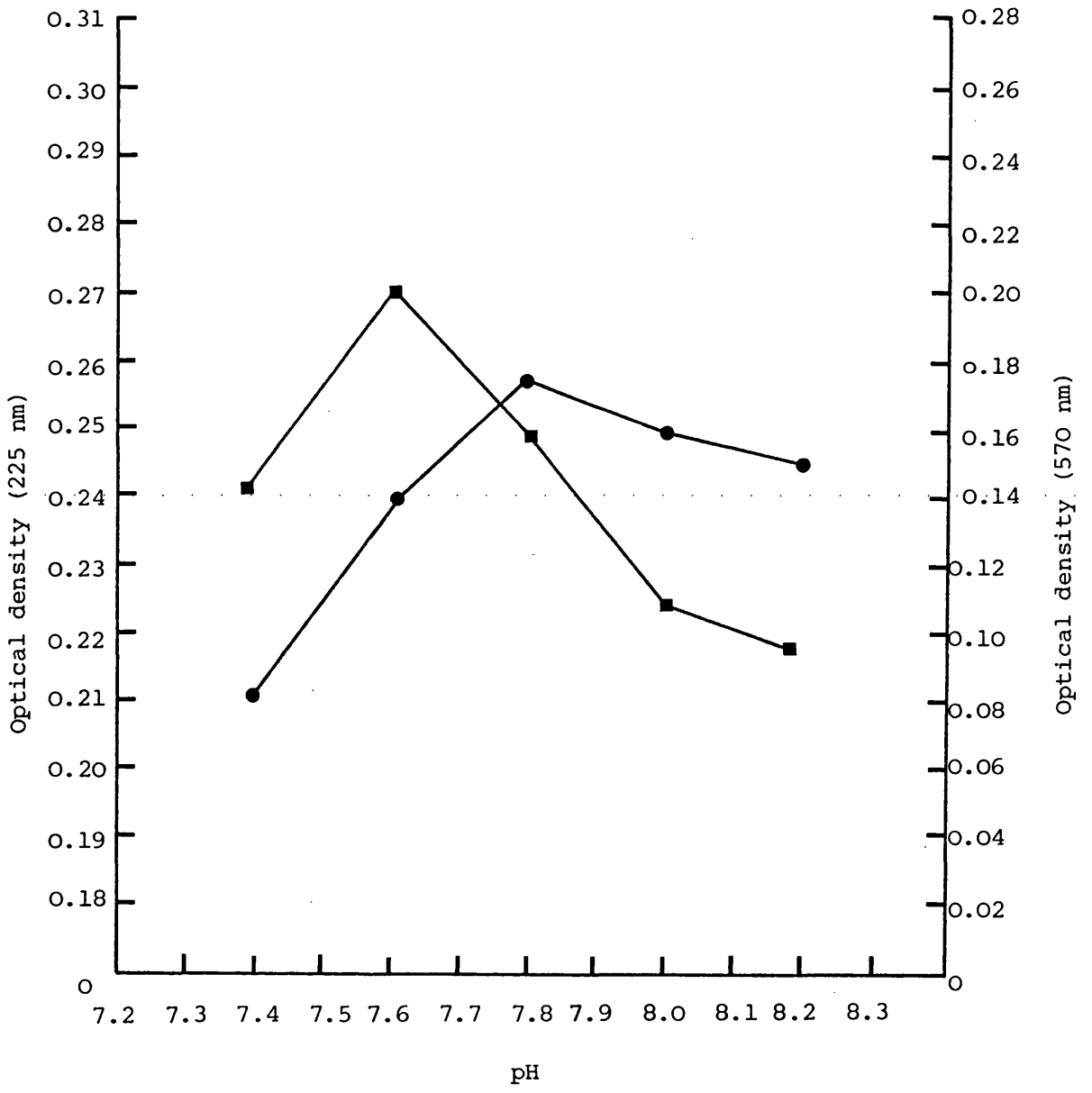


Figure 11. Determination of the pH optimum of pronase in tris HCl buffer. Measurements were made at 225 nm (●) and using ninhydrin at 570 nm (■).

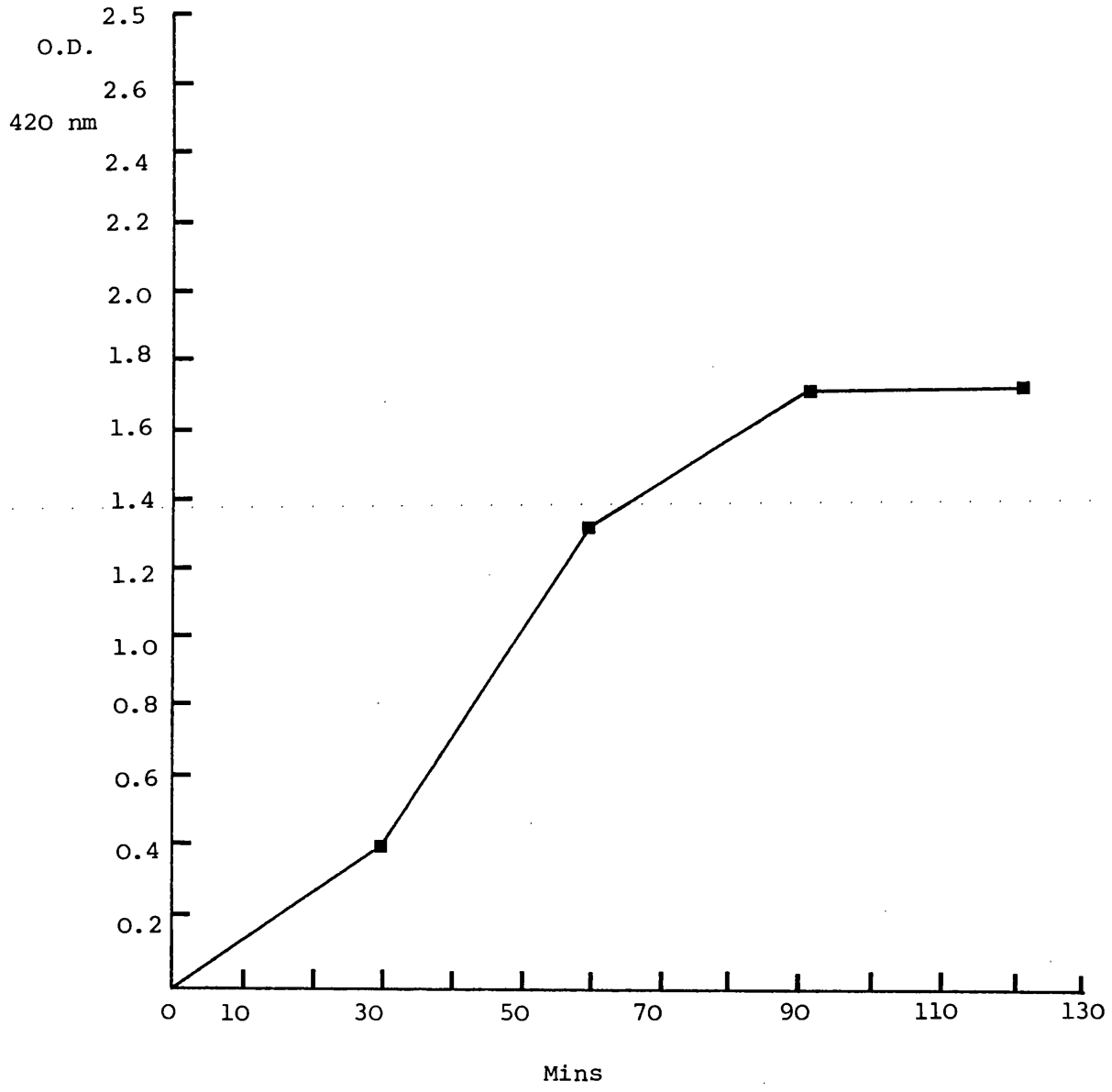


Figure 12. The release of hexose from membrane fragments by pronase digestion vs. incubation time, after correction for pronase auto-digestion.

Time	Protein concentration of supernatant (µg per ml)
1h	62
4h	71
24h	74

Table 4. Protein released by pronase digestion vs. incubation time.

Membrane fragments (10 mg protein detected by Lowry assay) were sonicated in an ice bath for two periods of 30s each at maximum power. Following sonication, these membrane fragments were digested with pronase as described above and centrifuged (100,000 x g. 1 h) to produce Extract I. This was then eluted from a column (2.5 x 100 cm) of Sephadex G50 using double distilled water, and the fractions freeze concentrated to 50 ml. The protein concentration of this solution totalled 5.6 mg. Correcting for the presence of pronase (500 µg) a figure of 5.1 mg protein recovered from Sephadex G50 following pronase digestion was obtained. Freeze drying 1 ml. of membrane fragment suspension gave a dry weight of 10.8 mg. Thus in 10 ml of suspension there was approximately 108 mg of membrane material, 10% of which was shown to be protein by Lowry assay.

Hexose, (assayed as described in the Methods section, p.81.) was released from membrane fragments concomitant with protein release (Figure 12). Table 5 shows a comparison of the degree of hexose (O.D. 420 nm) and protein (O.D. 750 nm) release from Extract I and from pronase incubated alone (Extract VI), under the same conditions.

Time	Extract I		Extract VI	
	750 nm	420 nm	750 nm	420 nm
1h	0.130	0.430	0.040	0.008
2h	0.160	0.430	0.020	0.008
4h	0.120	0.420	0.040	0.015
24h	0.120	0.430	0.070	0.030

Table 5. A comparison of the degree of release of hexose and protein from Extracts I and VI with time

It can be clearly seen that more hexose was present in the supernatant of Extract I following centrifugation (100,000 x g. 1h) than could be accounted for by pronase autodigestion alone.

Digestion using Immobilized Pronase (Extract II)

The pronase enzyme mixture was coupled to "Enzacryl" beads at a range of pH values prior to its use in the digestion of membrane frggments. Pronase (2 mg) in buffer (2 ml) was added to a stirred suspension of "Enzacryl" (40 mg) in 0.1M sodium phosphate buffer at four different pH values.The suspension was stirred (5h, $0^{o}C$) and centrifuged at 2,000 rev. per min for 10 min. The recovered solid was washed with 0.2 M sodium acetate buffer pH 5 (5 ml) followed by sodium acetate buffer pH 5 containing 1 M NaCl. The solid was isolated by centrifugation as before, and used immediately to digest membrane fragments under the conditions previously described. The recovery of soluble protein is shown in Table 6.

Sample	Protein release	% of Extract I
Extract I	0.500 mg per ml	100
Extract II (pH 6.0)	0.304 mg per ml	61
(pH 7.0)	0.300 mg per ml	60
(pH 7.6)	0.405 mg per ml	81
(pH 8.0)	0.322 mg per ml	65

Table 6. Protein release from the membrane fragments, a comparison between Extracts I and II.

2.3 Protein Release in the Absence of Pronase

Control experiments were performed in order to determine the extent to which soluble protein was released from membrane fragments by endogenous proteolytic activity, by treatment with NaCl, and by butanol extraction.

(i) Endogenous proteolysis (Extract III)

Membrane fragments, (approximately 10 mg protein) were suspended in tris-HCl buffer, pH 7.8 (5 ml) and incubated at 37°C for 30 min. The mixture was centrifuged (100,000 x g. 1h) and the supernatant assayed for protein using the method of Lowry (Methods Section p. 78).

(ii) Treatment with NaCl (Extract IV)

Membrane fragments (approximately 10 mg protein) were stirred with tris-HCl buffer, pH 7.8 containing 1 M NaCl at 22°C for 45 min and centrifuged (100,000 x g. 1h). The supernatant obtained was assayed for protein as above.

(iii) Butanol extraction (Extract V)

Membrane fragments (approximately 10 mg protein) in tris-HCl buffer pH 7.8 (5 ml) was stirred with n-butanol (3 ml) at 0°C for 20min. The mixture was centrifuged and the aqueous phase assayed for protein.

Protein release by each of the treatments detailed is compared with that released by Extract I, under conditions previously specified, in Table 7.

Conditions	Protein (mg per ml)	Protein release corrected for pronase concentration (mg per ml)
Extract I	0.95	0.85
Extract III	0.16	0.16
Extract IV	0.32	0.32
Extract V	0.27	0.27

Table 7. A comparison of protein release from membrane fragments in the presence and absence of pronase

2.4 Demonstration of the Proteolytic Activity of Electric Organ Homogenates

To each sample of homogenate (approximately 10 mg protein) was added commercially-prepared azocasein (10 mg) and the mixture was incubated at 37°C for 15 min. Undegraded azocasein was precipitated by the addition of 1% perchloric acid (1 ml) and the supernatant assayed at 285 nm to detect the release of the azo-group. Values of pronase equivalents were obtained by reference to a standard curve of pronase concentration vs. OD_{285nm}. Azo-group release from the substrate is proportional to the protease

concentration. A control containing perchloric acid was included to compensate for its presence in the assay mixture (Table 8).

Preparation	Protease activity concentration equivalent
H1	> 100 µg per ml
H2	12 µg per ml
H3	10 µg per ml

Key: H1 = homogenate in extraction medium plus NaN_3 (0.02%)

H2 = as H1 plus 1 mM EDTA

H3 = as H2 plus 0.1 mM PMSF

Table 8. Comparison of the proteolytic activity of electric organ homogenates

3. SEPHADEX G-50 FRACTIONATION OF MEMBRANE FRAGMENT PROTEOLYTIC DIGESTS

3.1 Soluble Pronase Digests (Extract I)

Membrane fragments (approximately 10 mg protein) in tris-HCl buffer containing 4 mM $CaCl_2$; pH 7.8 (50 ml) were incubated at 37°C for 30 min with a 10:1 (by weight) ratio of membrane protein to pronase. These conditions were chosen so as to afford a maximum yield of soluble protein (see preceding section). When incubation was completed, the digest mixture was centrifuged (100,000 x g. 1 h), the supernatant concentrated to half-bulk by freeze-drying and a sample (10 ml - 15 ml) was applied to the top of a column (2.5 x 100 cm) containing Sephadex G-50 in double-distilled water plus 0.02% NaN_3. Elution of this column with double-distilled water and monitoring at 280 nm using a Cecil Spectro-

photometer, gave the elution pattern shown in Figure 13. Assay of the column fractions by ninhydrin was found to be insufficiently sensitive, and continuous monitoring at 225 nm using a flow cell was not practically possible as very high extinction coefficients were obtained (Figure 14).

The same pattern was obtained from incubation periods ranging in time from 30 min. to 72h, although there was some variation in the sizes of individual peaks. Hexose was consistently associated with all the peaks obtained. Pronase autodigestion products were also demonstrated in the column eluate as follows: Pronase (4 mg)was dissolved in tris-HCl buffer, pH 7.8 and incubated at 37°C for 30 min. The digest was immediately frozen and then thawed after 24h and eluted from Sephadex G-50 using double-distilled water, with monitoring at 280 nm (Figure 15).

3.2 Elution Pattern of Extract III

Chromatography on Sephadex G-50 of material released by endogenous proteolysis gave the elution pattern shown in Figure 16. A comparison of the elution profiles given by Extracts I and III following incubation for 17h is shown in Figure 17.

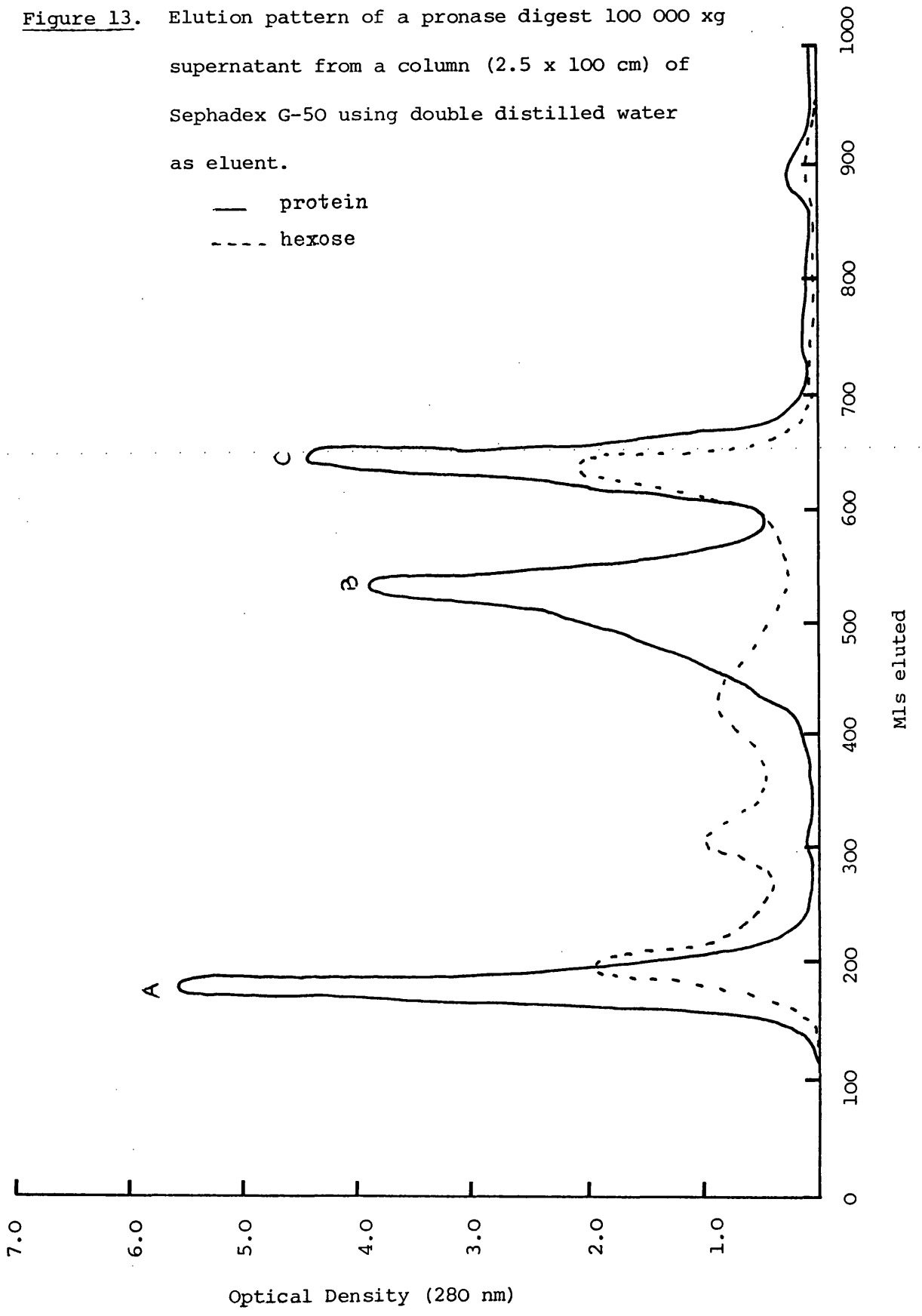
3.3 Elution Pattern of Extract IV

When 1 M NaCl solution extract supernatants were chromatographed, the pattern shown in Figure 18 was obtained. If fragments were first washed with tris buffer, extracted with 1M NaCl solution and then the pellet washed again with tris buffer before pronase treatment (Extract IV(a)), the pattern shown in Figure 19 was given. The breakthrough peak from this elution gave a single

Figure 13. Elution pattern of a pronase digest 100 000 xg supernatant from a column (2.5 x 100 cm) of Sephadex G-50 using double distilled water as eluent.

— protein

---- hexose



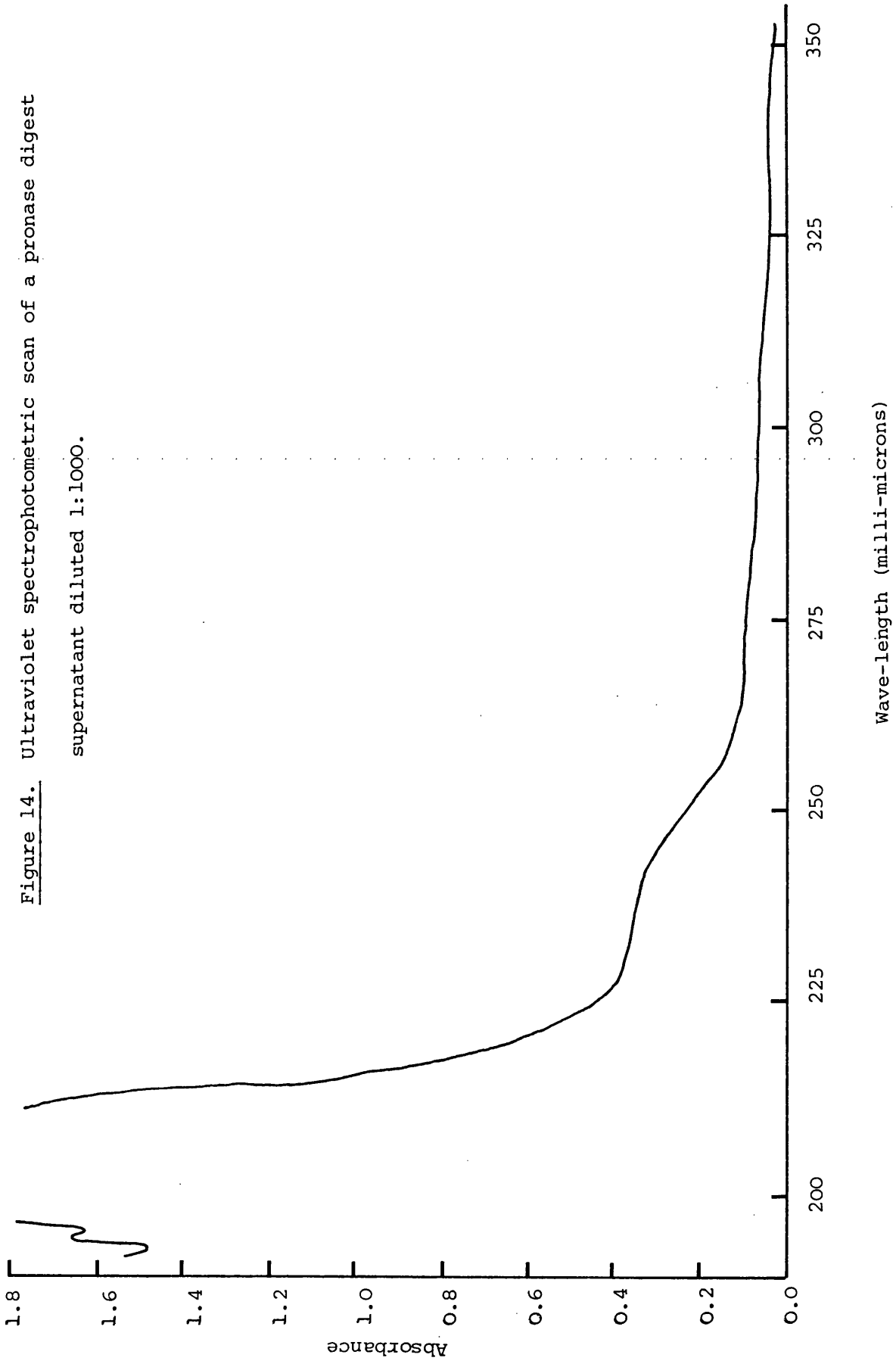


Figure 14. Ultraviolet spectrophotometric scan of a pronase digest supernatant diluted 1:1000.

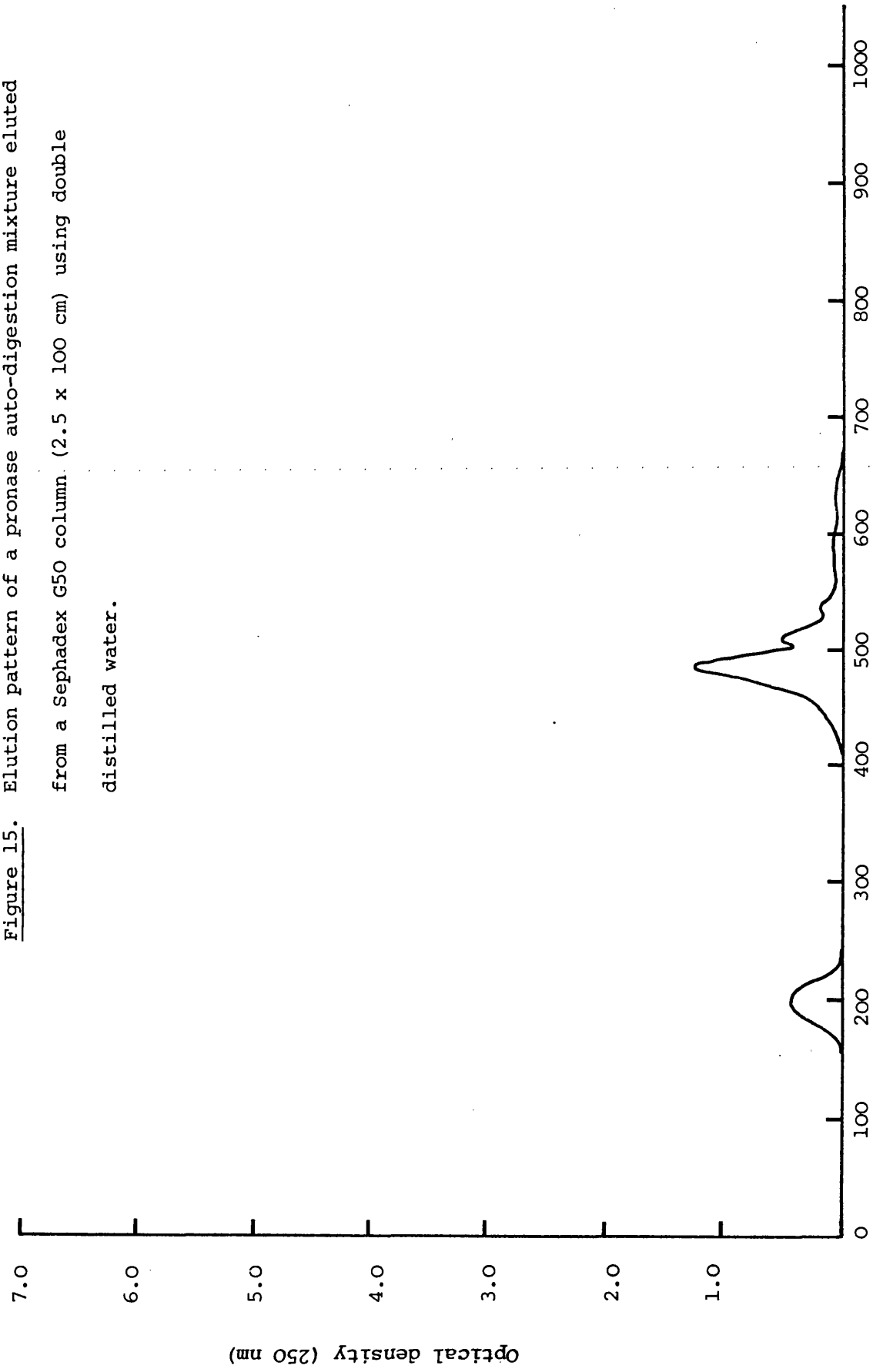


Figure 15. Elution pattern of a pronase auto-digestion mixture eluted from a Sephadex G50 column (2.5 x 100 cm) using double distilled water.

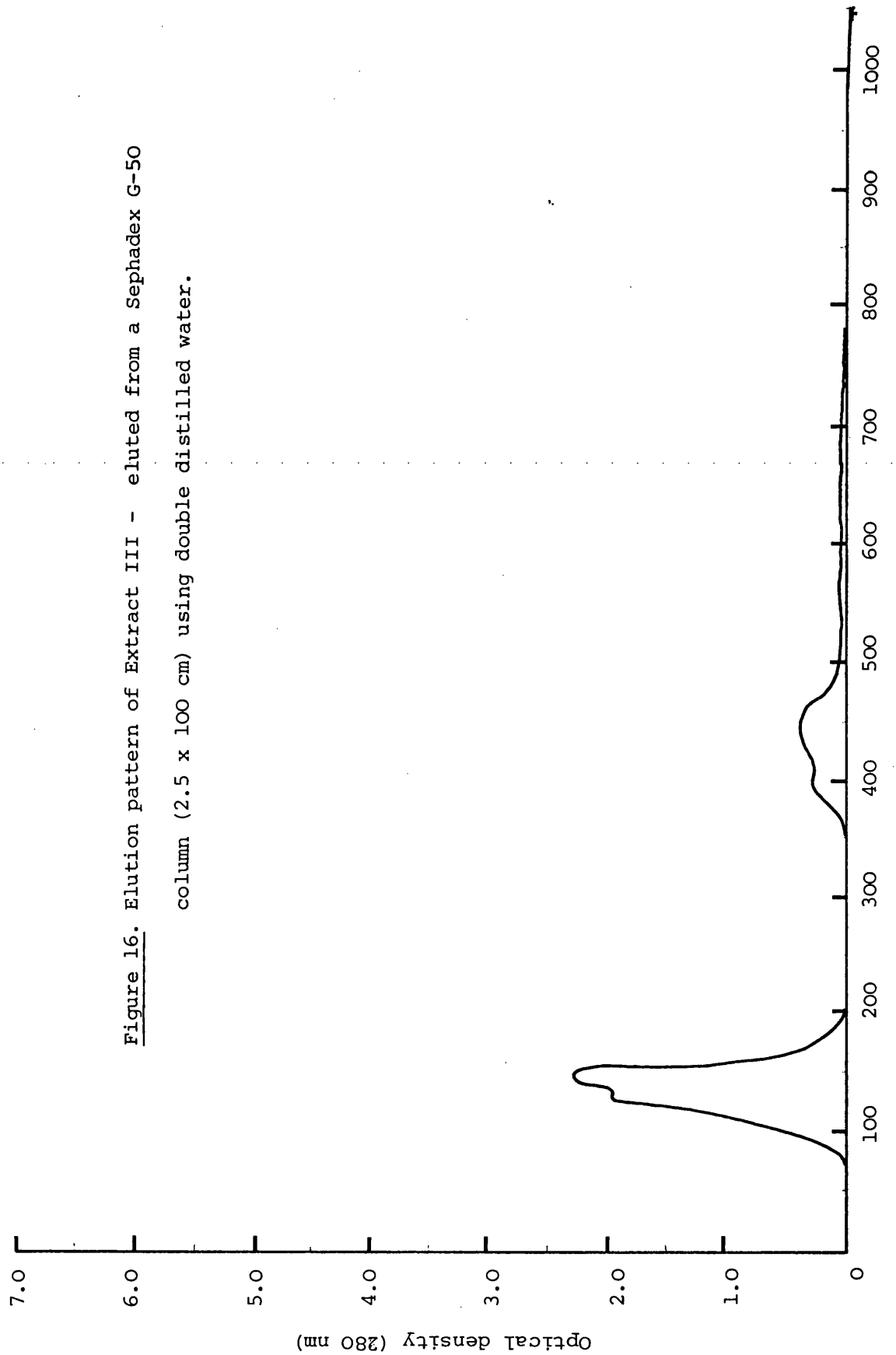


Figure 16. Elution pattern of Extract III - eluted from a Sephadex G-50 column (2.5 x 100 cm) using double distilled water.

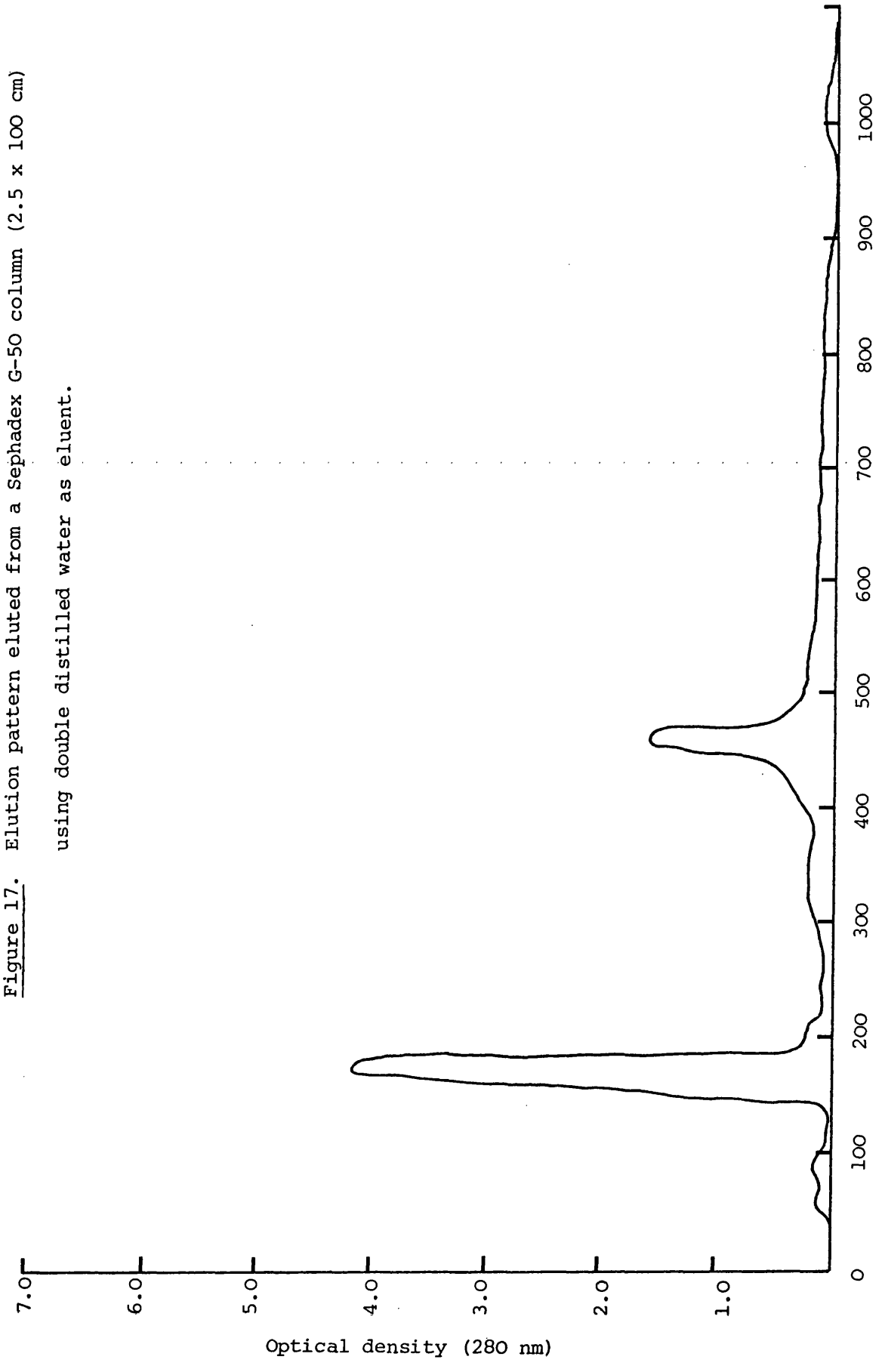


Figure 17. Elution pattern eluted from a Sephadex G-50 column (2.5 x 100 cm) using double distilled water as eluent.

component precipitate on rocket immunoelectrophoresis (Figure 20).

3.4 Calibration of Sephadex G-50 column using compounds of known molecular weight

The Sephadex G-50 column was calibrated by applying solutions of compounds of known molecular weight to the column and eluting using the conditions described above. The following compounds were employed:

Blue dextran	molecular wt >30,000 daltons
Myoglobin	molecular wt. 16,800daltons
Cytochrome C	molecular wt. 12,500 daltons.

The eluate was monitored at 280 nm, and the positions of the three peaks were correlated with both the fraction numbers and the elution volume (Figure 21).

4. SEPHADEX G-100 FRACTIONATION OF PROTEIN PEAKS EXCLUDED FROM SEPHADEX G-50

The cut-off ranges from G-100 are < 7,000 to 150,000 daltons. Fractions corresponding to Peak A from the Sephadex G-50 column eluate of Extract I(See Figure 13) were combined and concentrated to between 10 ml and 20 ml by freeze-drying, and applied to the top of a column (1.5 x 60 cm) of Sephadex G-100. Elution of the column with double-distilled water gave the pattern shown in Figure 22.

Figure 18. Extract IV; pattern obtained following elution from a Sephadex G50 column (2.5x100 cm) using double distilled water and monitoring at 280 nm.

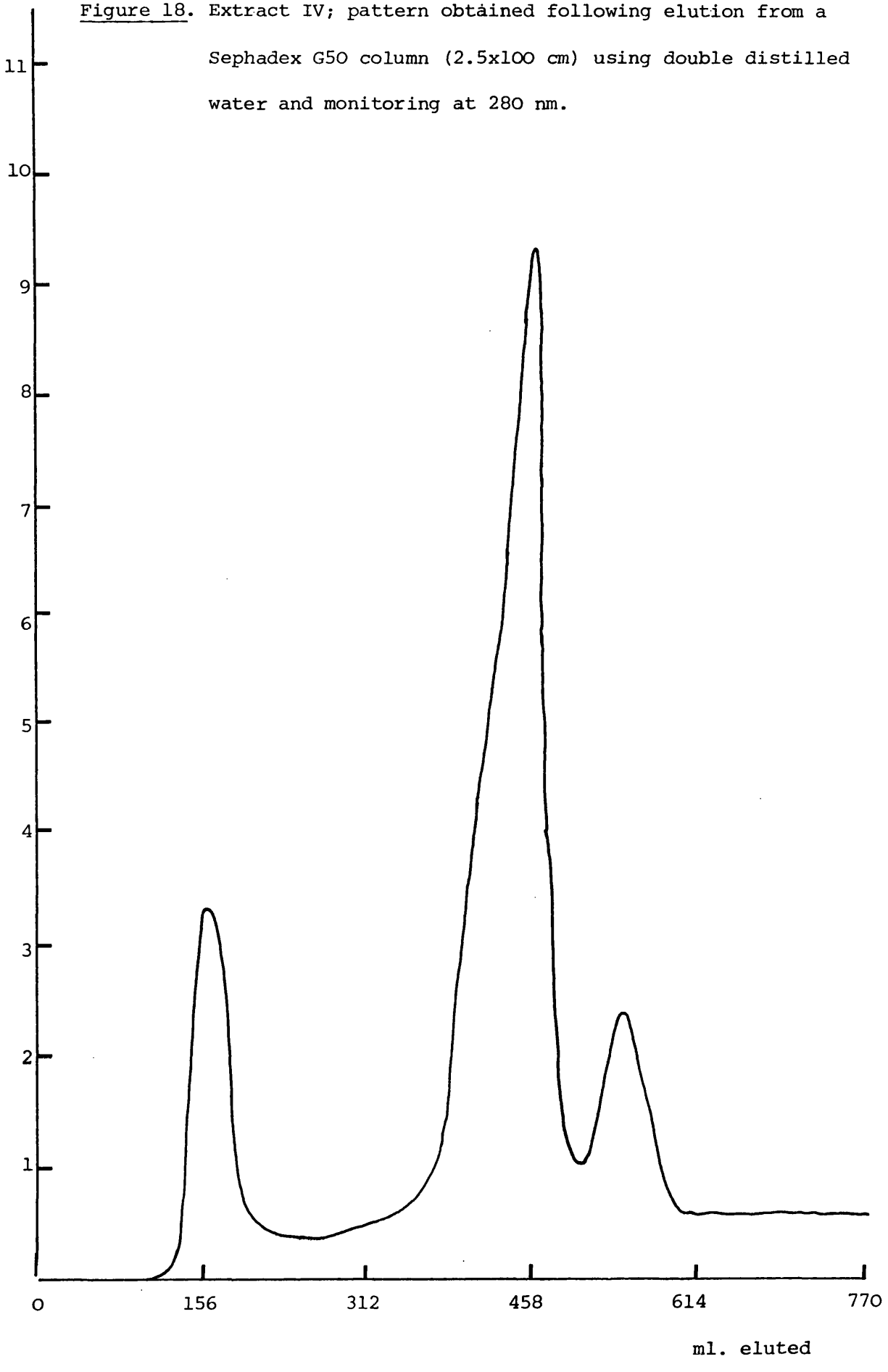
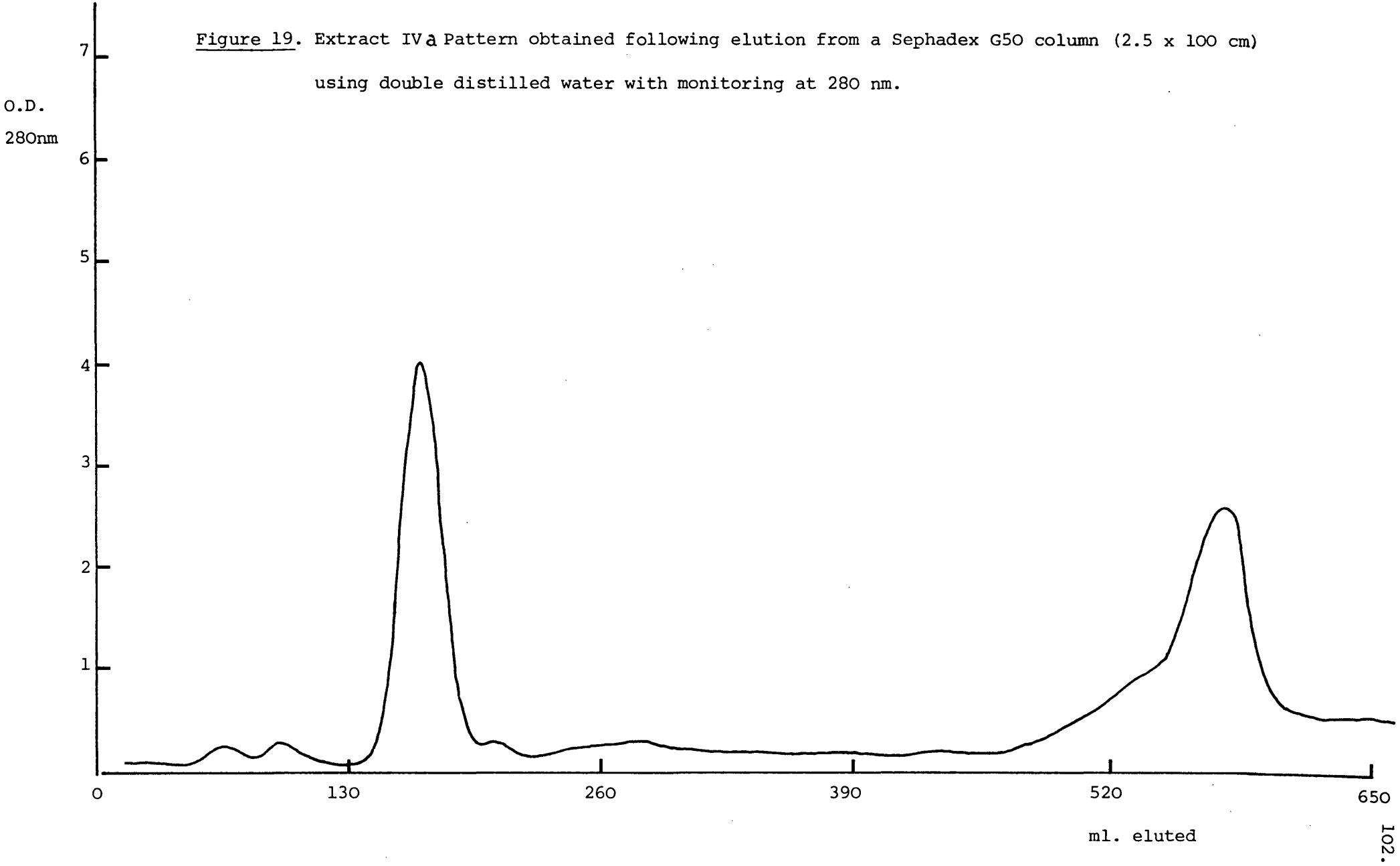


Figure 19. Extract IVa Pattern obtained following elution from a Sephadex G50 column (2.5 x 100 cm) using double distilled water with monitoring at 280 nm.



1 = Triton X-100 solubilized membrane fragments.

2 = Extract IV

3 = Excluded peak from Sephadex G50 chromatography of Extract IV

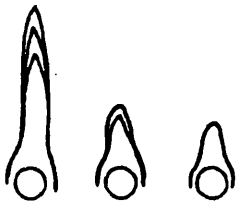


Figure 20. Extract IV. Rocket immunoelectrophoresis obtained with rabbit (anti-membrane) IgG (2% v/v). Electrophoresis performed 16h. at 2V per cm.

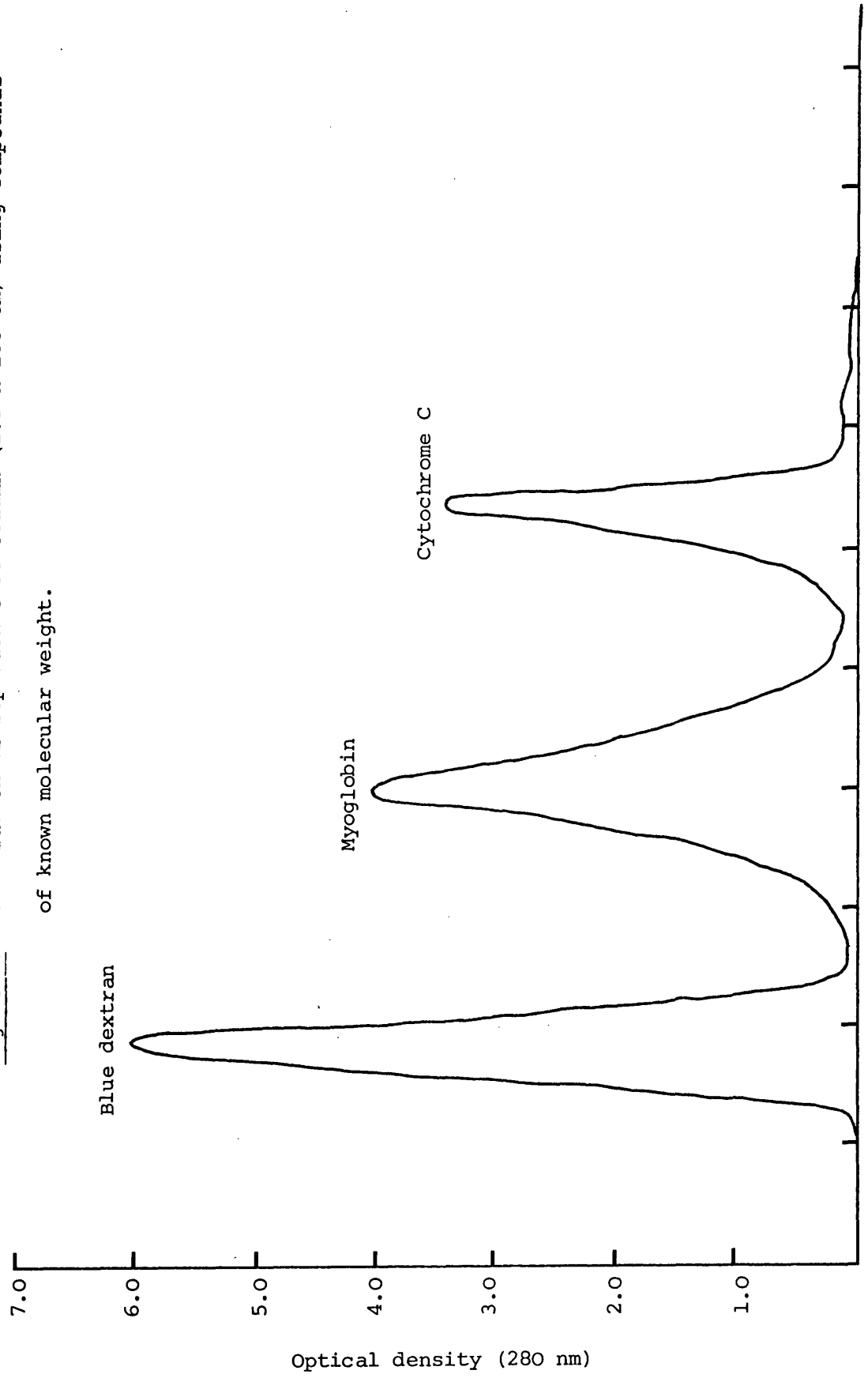


Figure 2b. Calibration of Sephadex G-50 column (2.5 x 100 cm) using compounds of known molecular weight.

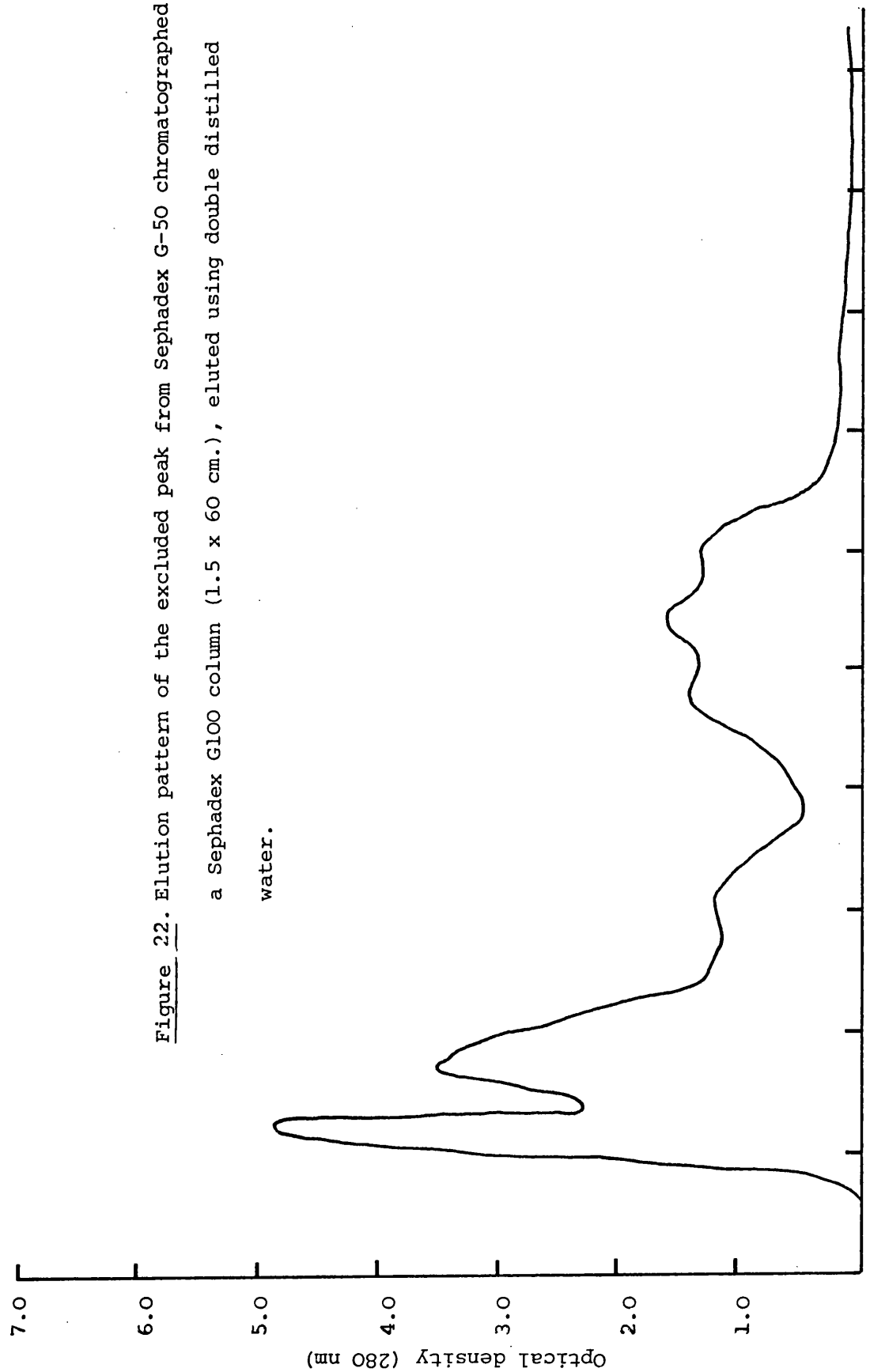


Figure 22. Elution pattern of the excluded peak from Sephadex G-50 chromatographed on a Sephadex G100 column (1.5 x 60 cm.), eluted using double distilled water.

5. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

5.1 Electrophoresis of Membrane Fragments on Polyacrylamide Gradient Gels

Acetylcholine receptor-rich membrane fragments affinity labelled with tritiated MBTA were kindly supplied by Professor J.P. Changeux of the Pasteur Institute, Paris. Electrophoresis of these fragments, together with fragments prepared as described above, was performed using sodium dodecyl sulphate (2%, w/v) as described in the Methods section (p. 72). The radioactively labelled gel shows 12 bands ranging in molecular weight from greater than 100,000 daltons, to less than 30,000 daltons and is compared with the gel obtained for the unlabelled fragments in Figure 23. Extraction and scintillation counting of the labelled gel was performed by incubating gel slices in H_2O_2 overnight at 50°C, adding scintillant and counting in a Hewlett Packard scintillation counter. This showed that the radioactive affinity label was concentrated in bands numbered 8 and 9, which had approximate molecular weights of 60,000 daltons and 40,000 daltons respectively. Figure 24 shows the concentration of radioactive label in the gel compared to the positions of protein standards of known molecular weight. Thus the area of highest concentration is found in a position just ahead of enolase, which in its reduced form has a molecular weight of 41,000daltons, corresponding to a polyacrylamide concentration of 10%.

5.2 Disc Polyacrylamide Gel Electrophoresis

Electrophoresis was performed as described in the Methods (p. 74). The following samples were examined using this technique.

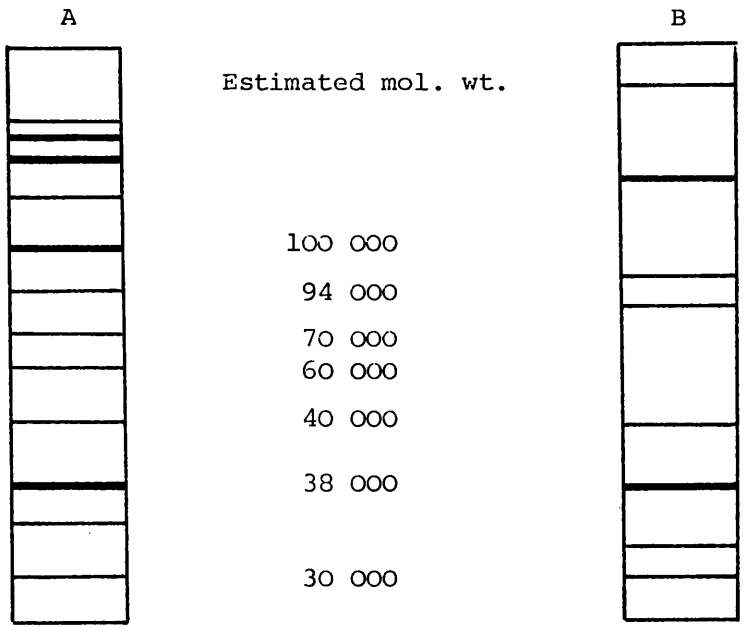


Figure 23. (Top) Comparison of PA disc gels of membrane fragments labelled with ^{3}H MBTA (A) and unlabelled (B).

(Bottom) Graph of Mr (protein standards ■-■) and cpm (● - ●) vs. gel band number following PAGGE of ^{3}H MPTA-labelled membrane fragments.

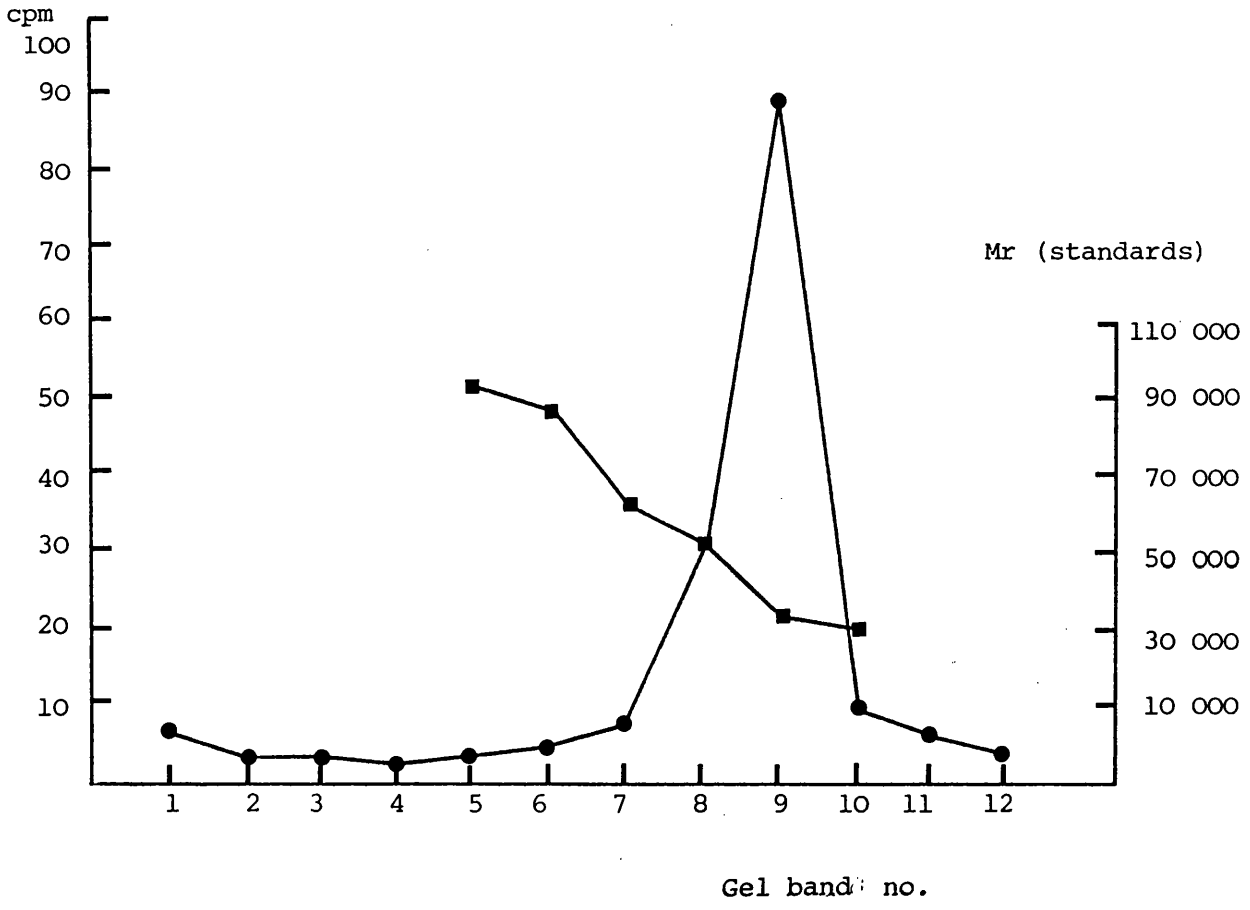


Figure 24. Diagram showing the concentration of radioactive label in the gel compared to the positions of protein standards of known molecular weight.

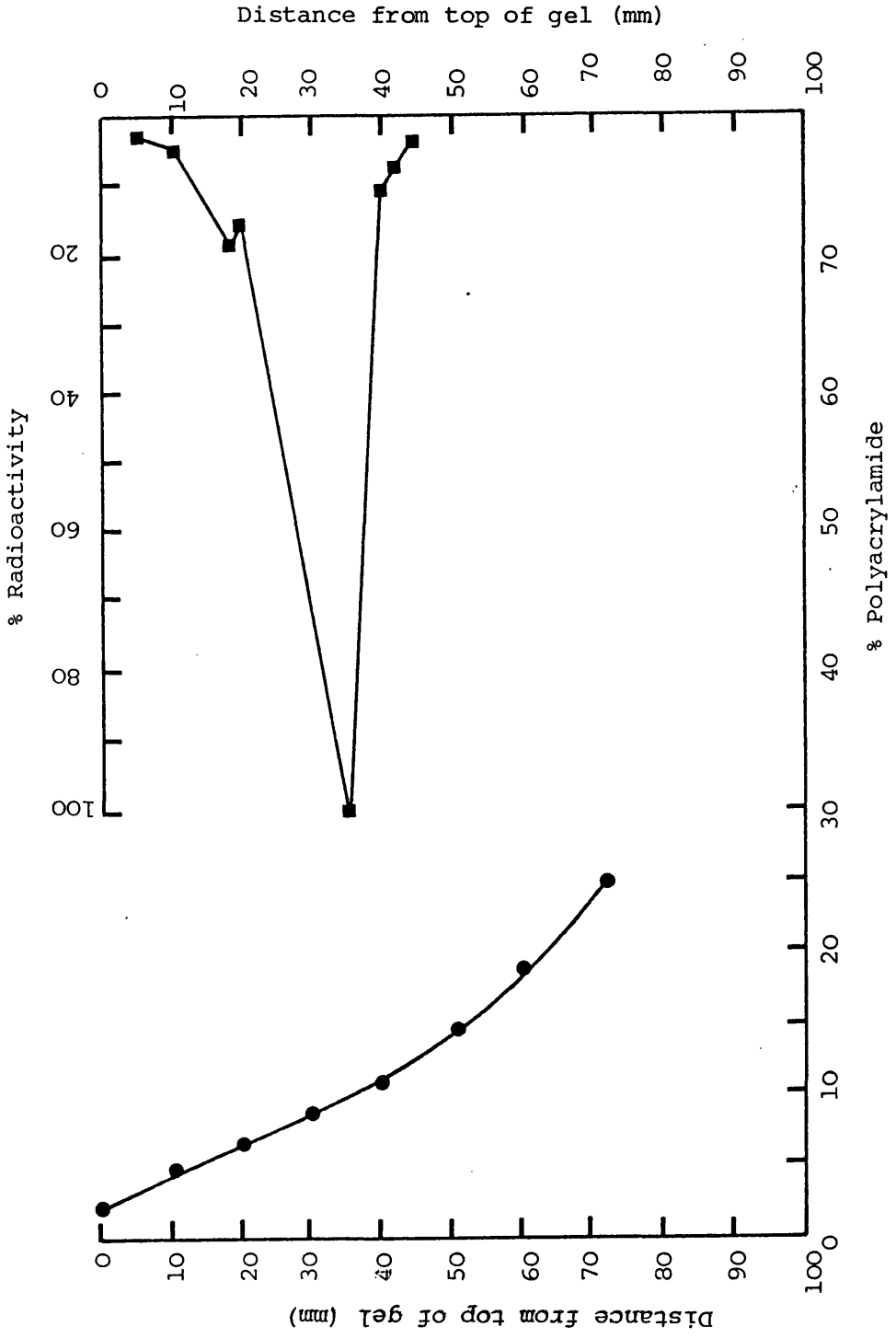

Distance from top of gel (mm)
0
10
20
30
40
50
60
70
80
90
100
% Radioactivity
20
40
60
80
100
% Polyacrylamide
70
60
50
40
30
20
10
0
Distance from top of gel (mm)

Figure 24.

(i) Protein standards of known molecular weight (Figures 25 and 26)

(ii) A Triton X-100 solution (100,000 x g. supernatant) of membrane fragments (Figure 27).

(iii) Extract IV (Table 9).

(iv) Extract VI (Figure 28).

(v) The column fractions obtained from Sephadex G-50 chromatography of Extract I (Figures 29-32).

(vi) The excluded peak from Sephadex G-50 chromatography of Extract IV (Table 9).

Band numbers	Mobility	Log MW	MW from calibration curve
1	25.6		>100,000 daltons
2	32.6		" "
3	44.2		" "
4	48.8		100,000 daltons
5	60.5	4.66	46,000 daltons
6	65.1	4.53	34,000 daltons
7	76.7	4.23	17,000 daltons
8	88.4	3.92	8,500 daltons
1*	51.2	4.93	85,000 daltons

Table 9. PAGE of Extract IV, and the excluded peak from Sephadex G-50 chromatography of Extract IV(*)

1.BSA (68000)
2.YADH (37000)
3.Trypsin (23300)
4.Myoglobin (18000)
5.Cytc. (12500)

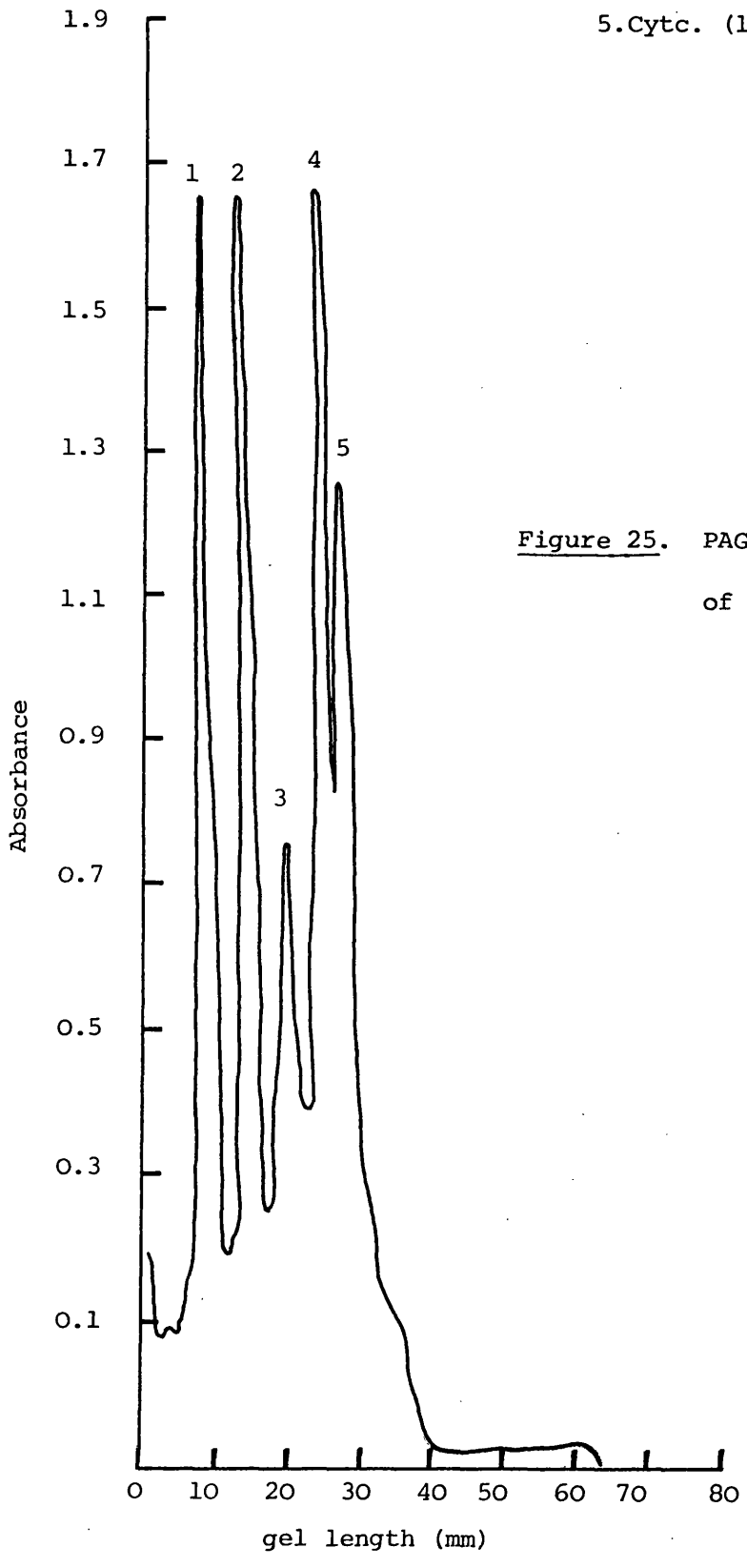


Figure 25. PAGE of protein standards of known molecular weight.

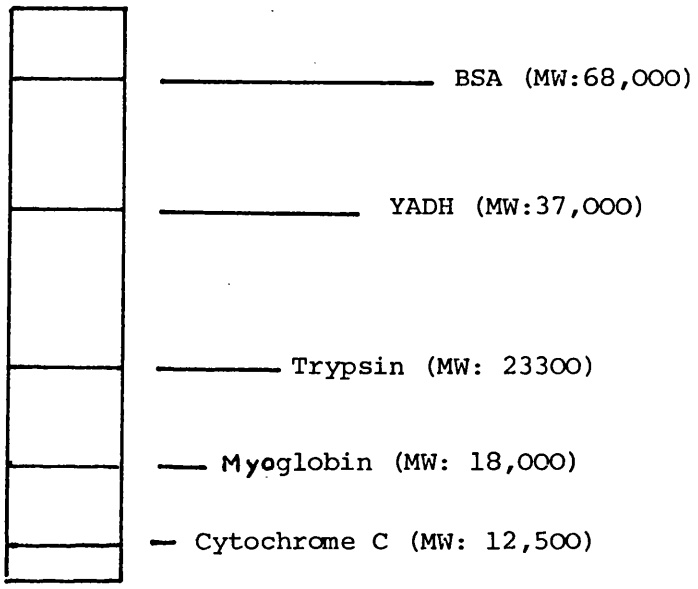
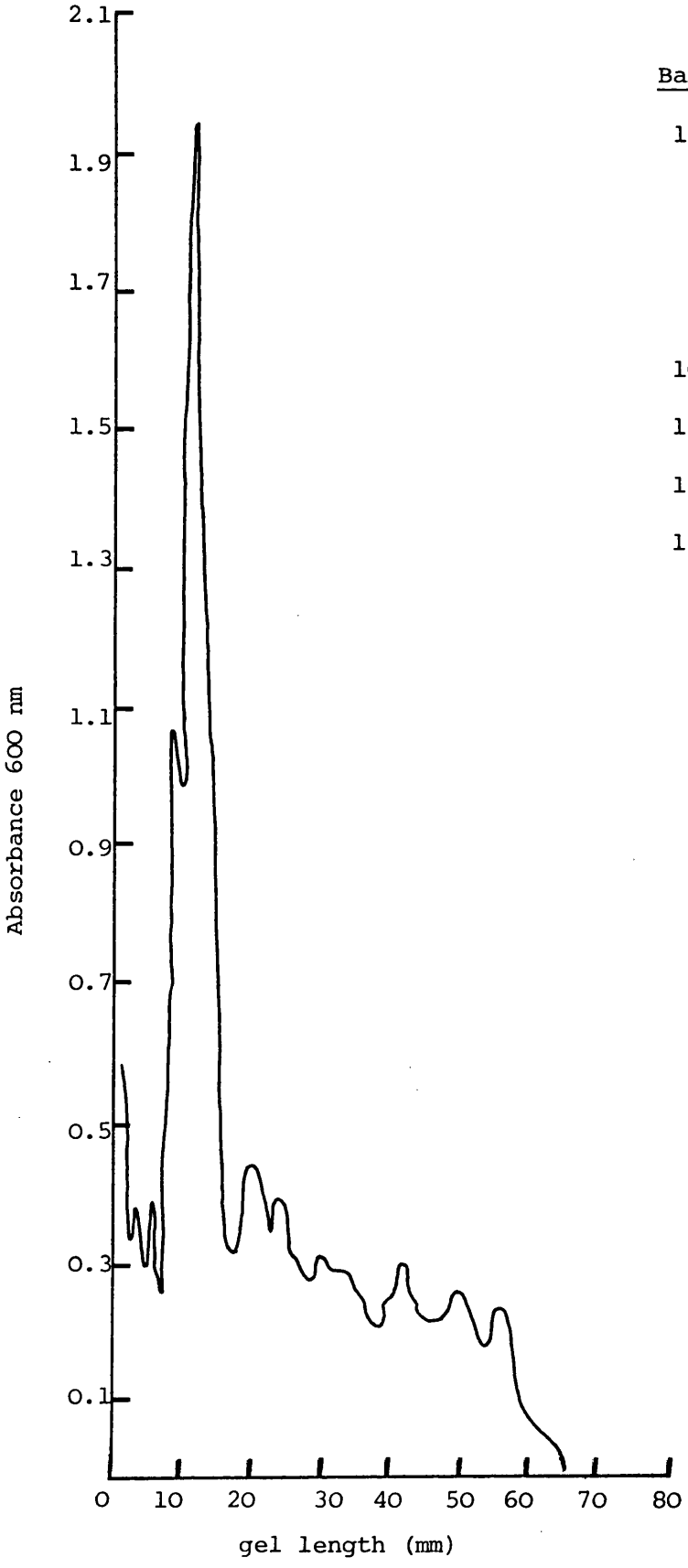


Figure 26. Illustration showing relative positions of protein standards used to calibrate disc gels for PAGE. The running conditions were as detailed in the Methods Section

Figure 27. Disc PAGE trace of a Triton X-100 (1% v/v) solution (100,000 x g. supernatant) of membrane fragments.



Band	Mr.
1-6	80000
7	66000
8	48000
9	40000
10	29000
11	22000
12	15000
13	12000

Figure 28 Disc PAGE trace of Extract VI.

band	Mr.
1	50000
2	22000
3	15000
4	ca. 4000
5	"
6	"

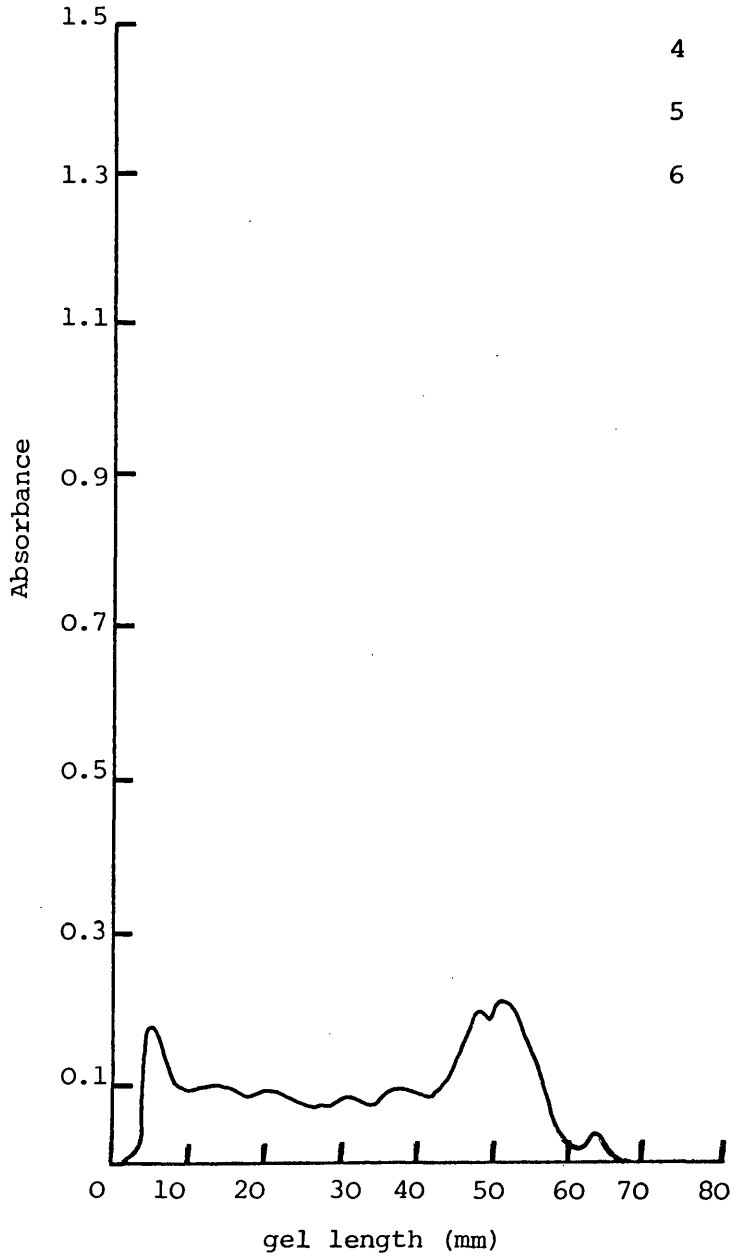
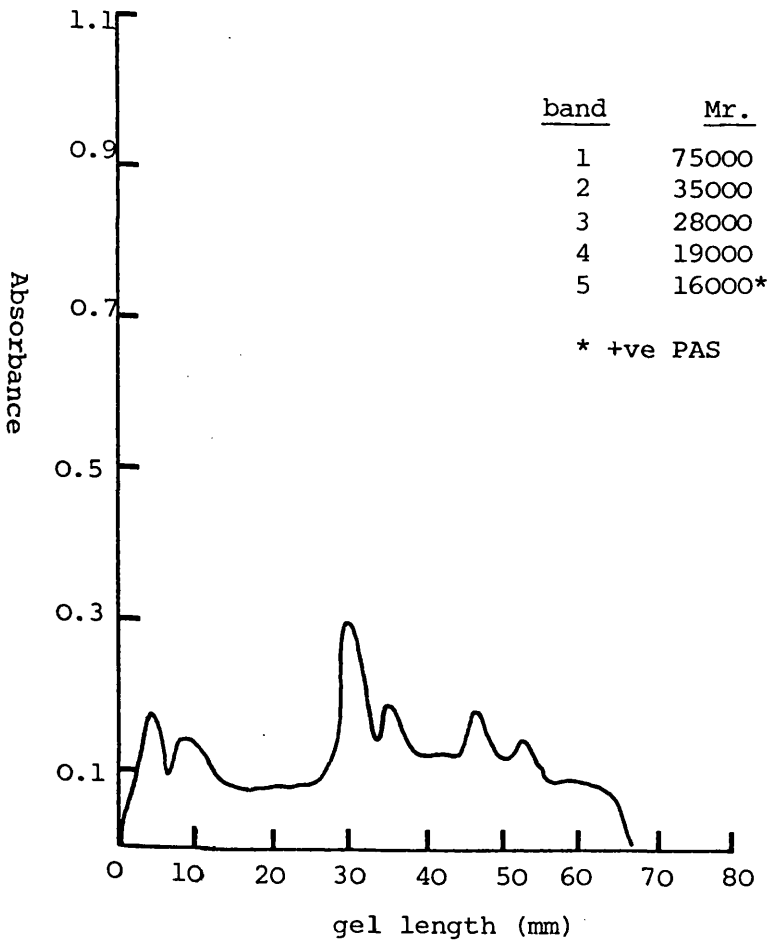


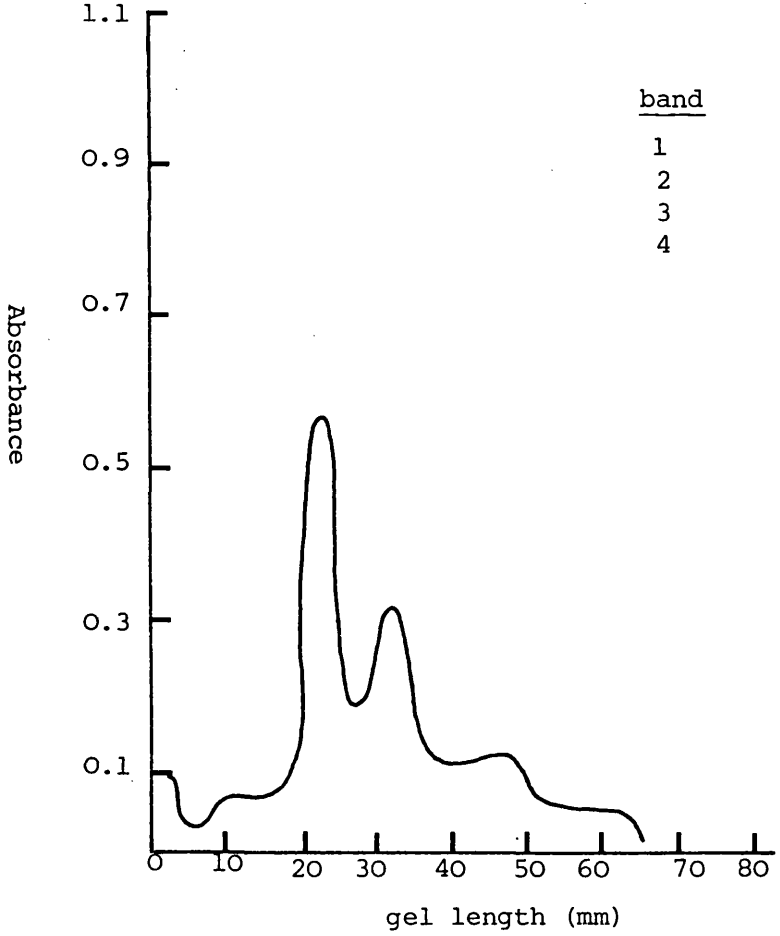
Figure 29 Disc PAGE trace of fractions 10-22 from Sephadex G50 column (2.5x10 cm) chromatography of Extract I.



band	Mr.
1	75000
2	35000
3	28000
4	19000
5	16000*

* +ve PAS

Figure 30 Disc PAGE trace of fractions 23-27 from Sephadex G50 column (2.5x100 cm) chromatography of Extract I.



band	Mr.
1	80000
2	70000
3	28000
4	20000

Figure 31 Disc PAGE trace of fractions 28-32 from Sephadex G-50 column (2.5x100 cm) chromatography of Extract I.

band	Mr.
1	28000
2	3500

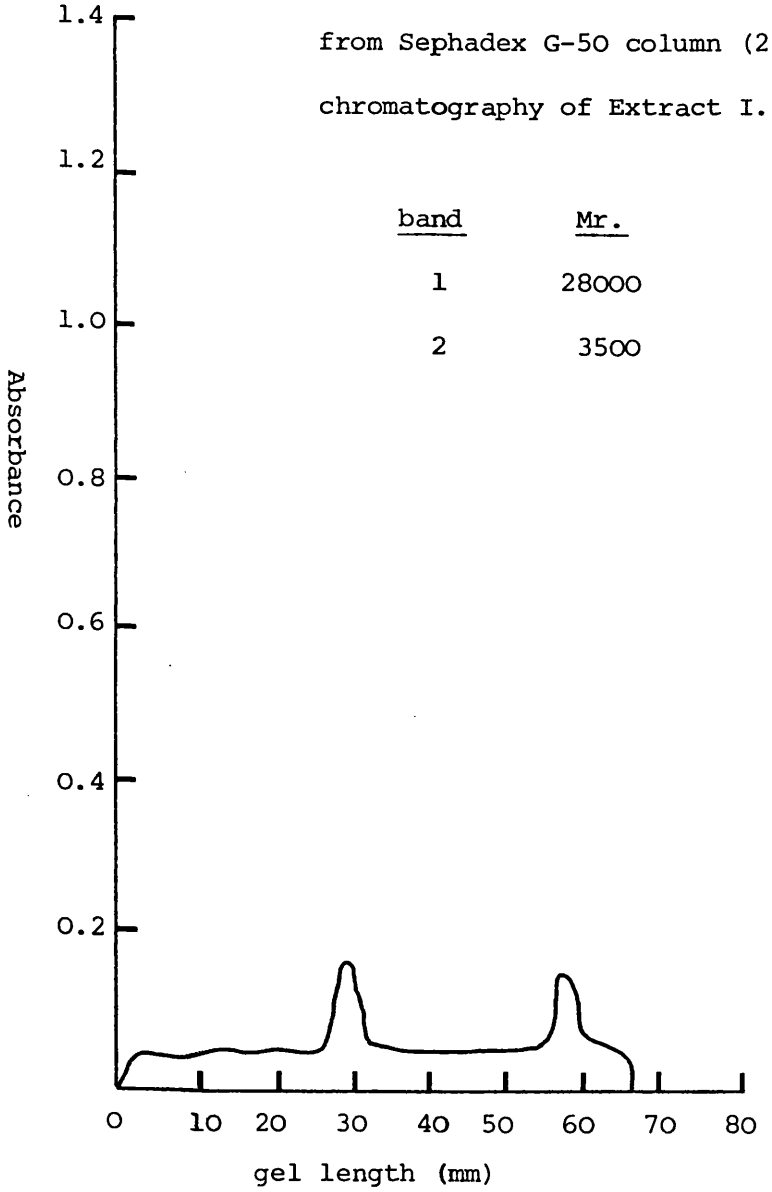
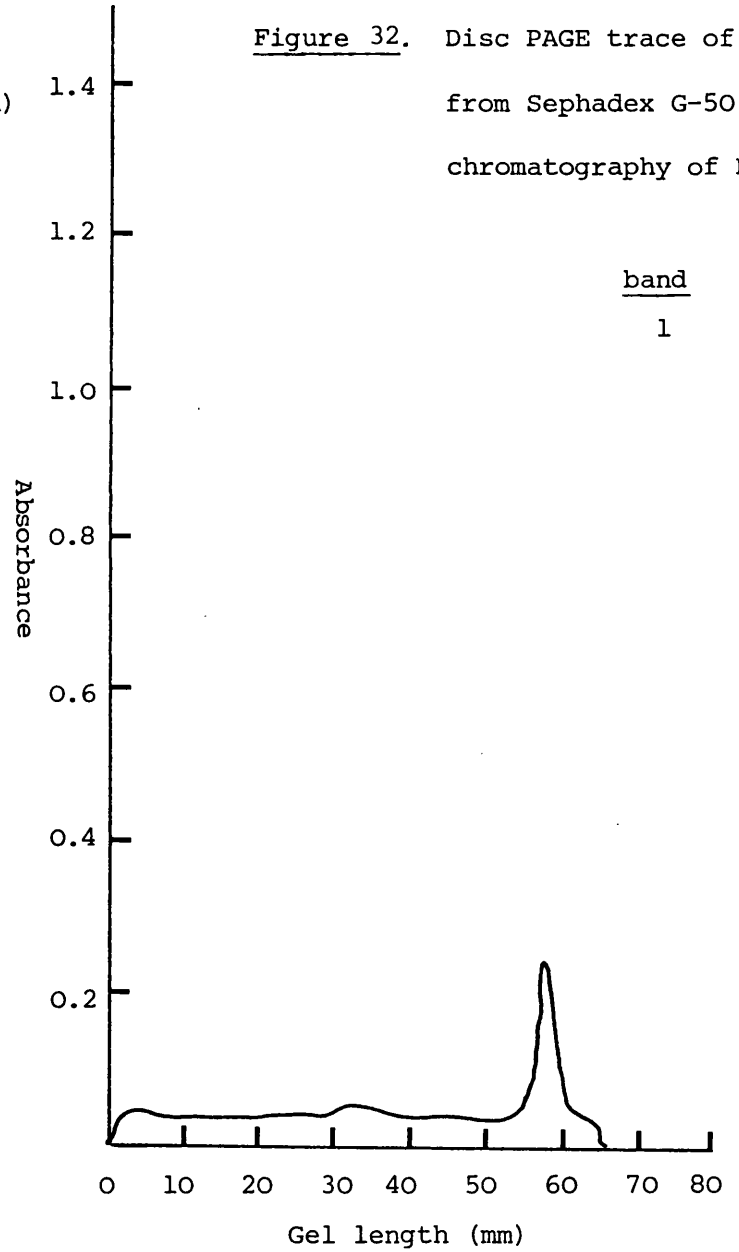


Figure 32. Disc PAGE trace of fractions 33-50 from Sephadex G-50 column (2.5x100 cm) chromatography of Extract I.

band	Mr.
1	3500



6. ACETYLCHOLINE-RECEPTOR-RICH MEMBRANE FRAGMENTS AS ANTIGENS

6.1 Immunoelectrophoretic Analysis of Detergent Solubilized Membrane Fragments

Rocket immunoelectrophoresis of membrane fragments solubilized in Triton X-100 (1 mg protein per ml) against rabbit antibody to Torpedo membrane preparations produced a pattern which indicated the presence of at least four, separate antigenic components (Figure 33) and was consistently repeatable. These assays were quantified by plotting distance migrated by each antigenic component of the rocket precipitate against the amounts of solubilized fragments in serial dilution. When plotted in this manner, each component gave a straight line (Figure 34) indicating that the distance migrated by the precipitated components is proportional to the concentration of antigen solution applied over a range of dilutions.

Two dimensional immunoelectrophoresis of the same sample showed a pattern (Figure 35) containing six peaks, three of which (4, 5 and 6) were very diffuse.

6.2 Antigenicity of Receptor Depleted Detergent-Solubilized Membrane Fragments

Detergent solubilized receptor-depleted membrane fragments were generously supplied by Dr. T. Barkas and were prepared by eluting a Triton X-100 solution (1%, v/v) of membrane fragments from a column of Sepharose 4B, to which α bungarotoxin had been conjugated by reaction with cyanogen bromide. The column eluate (ie. that material which did not bind to the column)

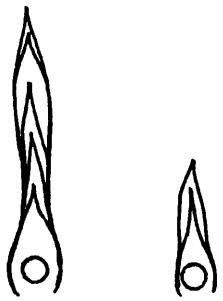


Figure 33. Rocket immunoelectrophoresis of Triton X-100 (1% v/v) solubilized membrane fragments (1); and non-solubilized membranes (2) vs. rabbit antibody (2% v/v) in agarose (1% w/v) gel.

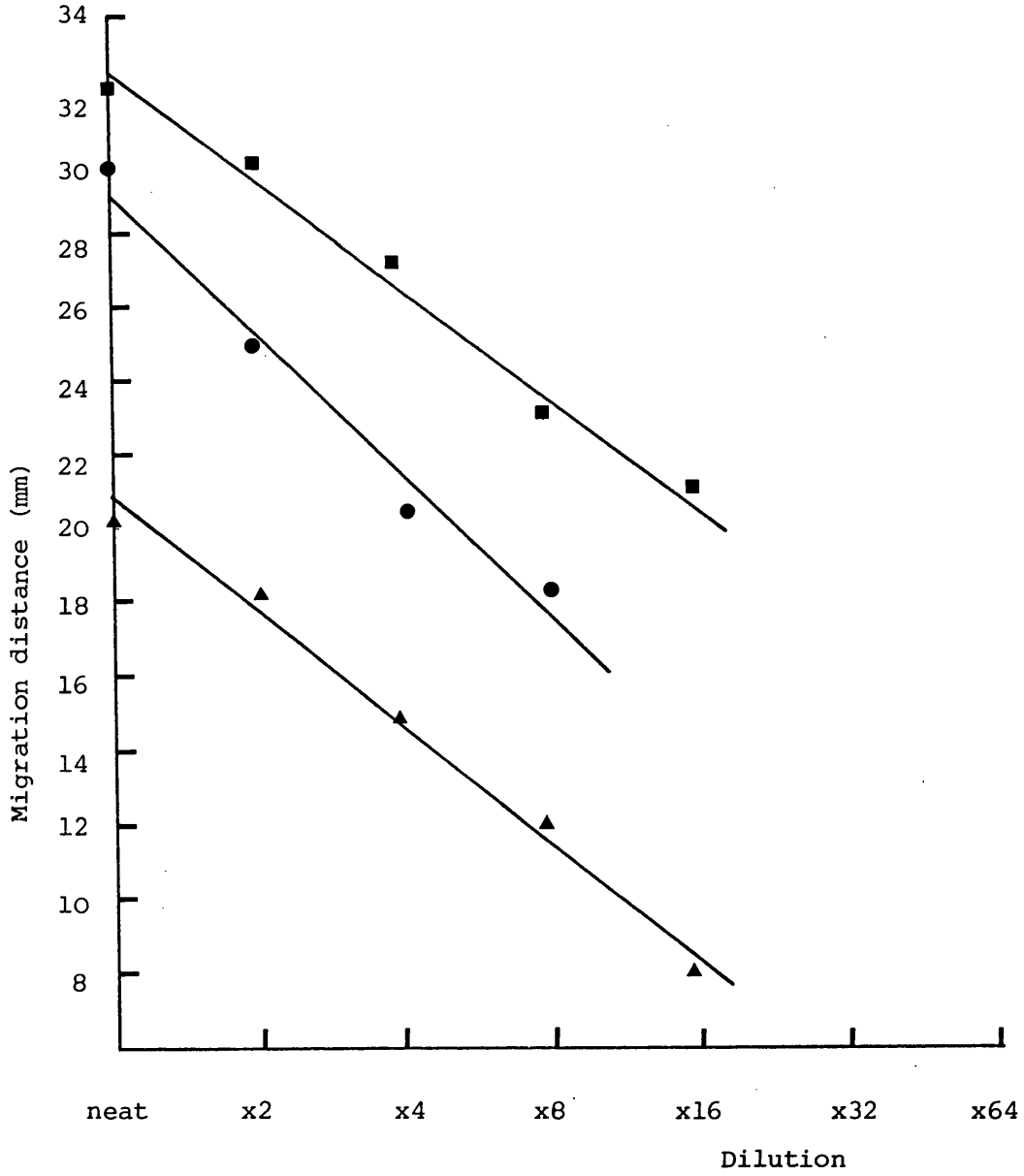


Figure 34. Plot of migration distance vs. the dilution factor for three components found on rocket immunoelectrophoresis of Triton solubilized membranes vs. rabbit antibody.

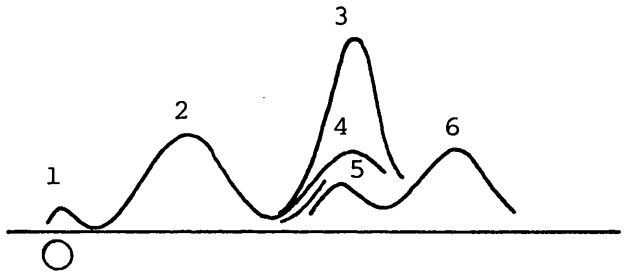


Figure 35. Two-dimensional immunoelectrophoresis of a sample of detergent (Triton X-100) solubilized membrane fragments against rabbit antibody (2%, v/v). Electrophoresis performed at 10 V per cm (1st dimension) for 1h. and 2V per cm (2nd dimension) for 16h.

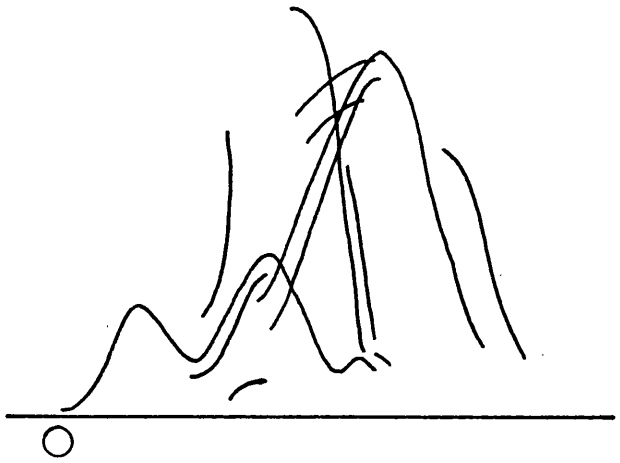
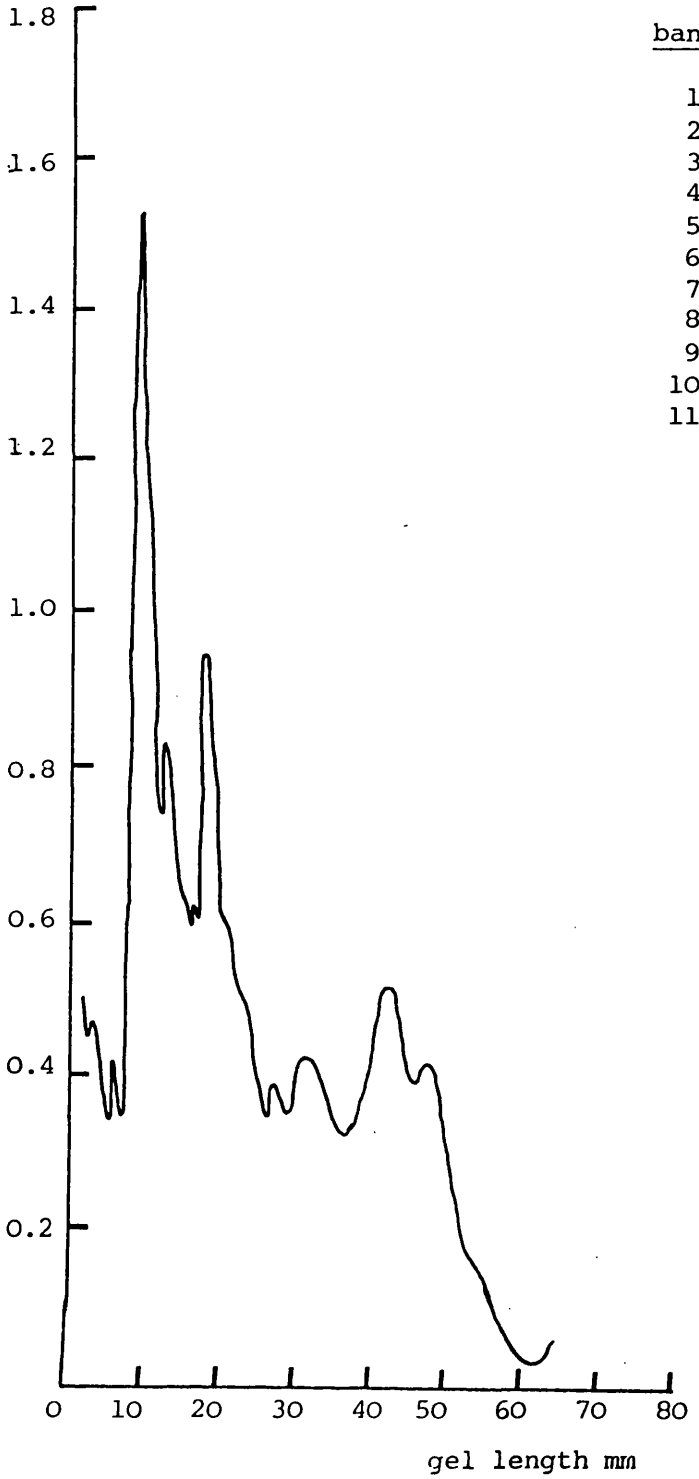


Figure 36. Two-dimensional immunoelectrophoresis of a sample of receptor-depleted, detergent solubilized membrane fragments against rabbit antibody (2%, v/v). Electrophoresis performed at 10 V per cm (1h) for 1st dimension and 2V per cm (16h.) for the second dimension.

Figure 37. Disc PAGE trace of Triton X-100 solubilized membrane fragments depleted of the receptor content by elution from a column of Sepharose 4B to which α Bgt had been coupled.



band	Mr
1	80000
2	"
3	78000
4	66000
5	54000
6	48000
7	42000
8	35000
9	33000
10	30000
11	23000

was assayed by crossed immunoelectrophoresis (Figure 36) and also by polyacrylamide gel electrophoresis (Figure 37).

6.3 Immunoelectrophoretic Analysis of Proteolytic Digests

Membrane fragments were prepared as previously described and incubated with pronase (1 mg) in 50 mM tris-HCl buffer pH. 7.8 containing 4 mM $CaCl_2$, for periods of 30; 60 and 90 min. Each sample was then centrifuged at 10,000 rev per min and the supernatants were assayed by double diffusion in agarose gel (1%) against rabbit antibody to membrane fragments (Figure 38). One of the two precipitates obtained showed a reaction of identity with theprecipitate given by membrane fragments solubilized in Triton X-100 (1%). All three digests contained hexose (Figure 39).When the samples were centrifuged at 100,000 x g. for 1h and the supernatants assayed in the same manner, single-line precipitates were obtained, which continued to give a reaction of identity with detergent solubilized membrane fragments (Figure 40). Two dimensional immunoelectrophoresis of Extract I gave the pattern shown in Figure 41.

6.4 Double Diffusion Assays using Sheep Anti-AChR Serum

Sheep anti-AChR serum was a gift from Dr. T. Barkas. When used in double diffusion assays with Extract I, the excluded peak from Sephadex G-50 chromatography of Extract I; Extract III; a Triton X-100 solution of membrane fragments and a Triton X-100 solution of receptor depleted membrane fragments, the precipitin pattern shown in Figure 42 was obtained. The precipitate given by Extract III was faint in comparison with that given by the other samples.

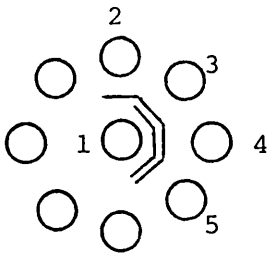


Figure 38. Double diffusion analysis of proteolytic digest time course: 30 min (3); 60 min (4); 90 min (5). Triton solubilized membrane fragments (2) were included as a control. Rabbit antibody (10 µl) was contained in well 1.

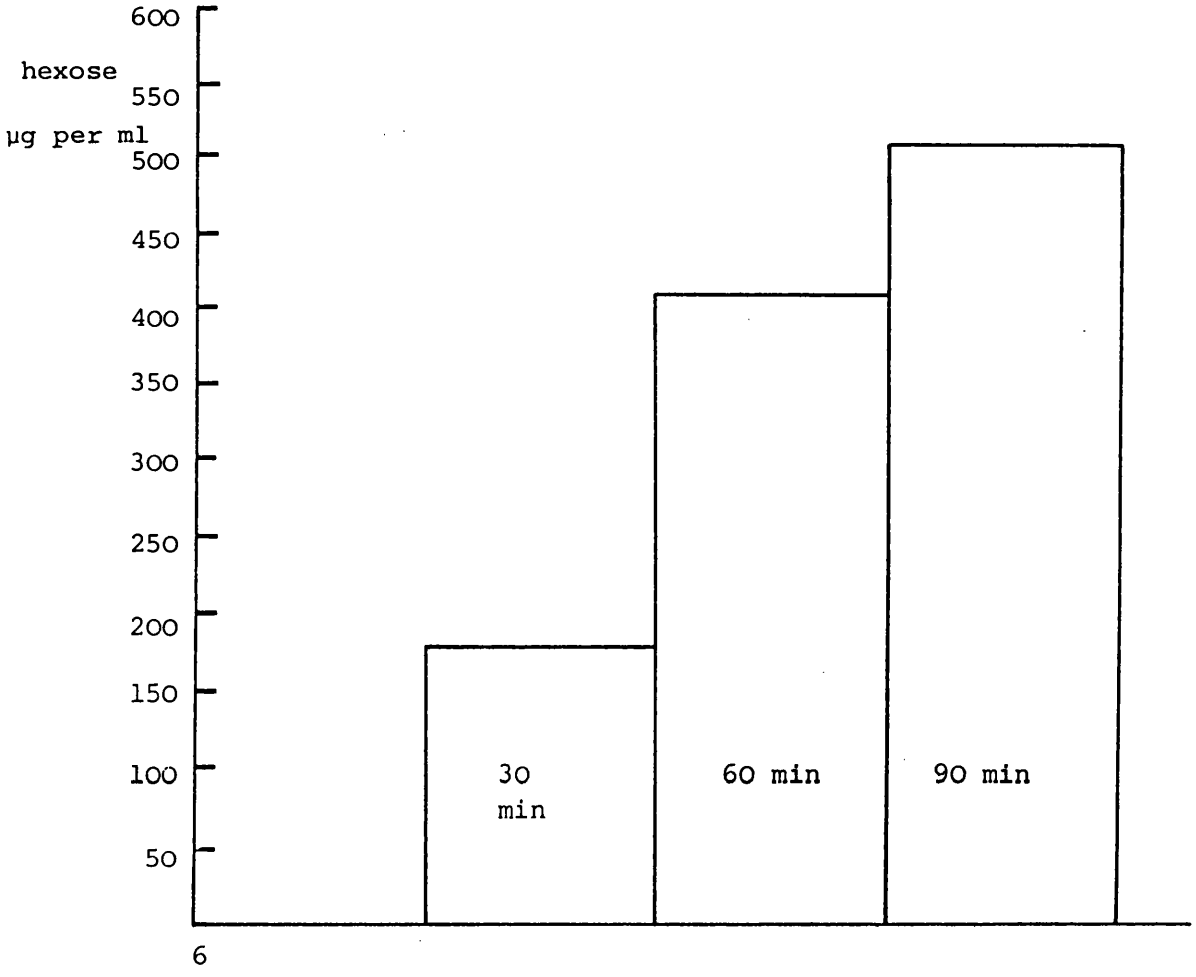


Figure 39. Amounts of hexose contained in proteolytic digest time course.

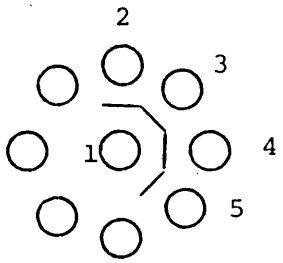


Figure 40. Double diffusion analysis of proteolytic digest time course supernatants (100,000 x g., 1 h.) 30 min (3); 60 min (4); 90 min (5) compared with Triton solubilized membrane fragments (2), using rabbit antibody (1).

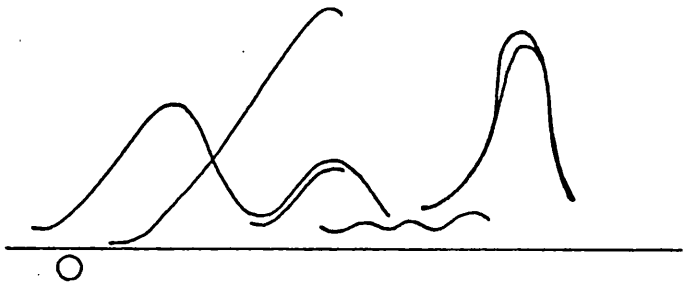
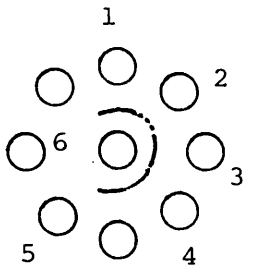


Figure 41. Two dimensional immunoelectrophoretic analysis of Extract I. Running conditions: rabbit antibody incorporated into agarose gel at 2% (v/v), electrophoresis performed for 1h. 10 V per cm (1st dimension, 16h. at 2V per cm (2nd dimension).



1 = Extract I
2 = Extract III
3 = Triton solution of membrane fragments
4 = excluded peak from G-50 chromatography of Extract I.
5 = Triton solution of receptor depleted membrane fragments
6 = sheep anti AChR serum

Figure 42. Double diffusion assay using sheep anti-AChR serum.

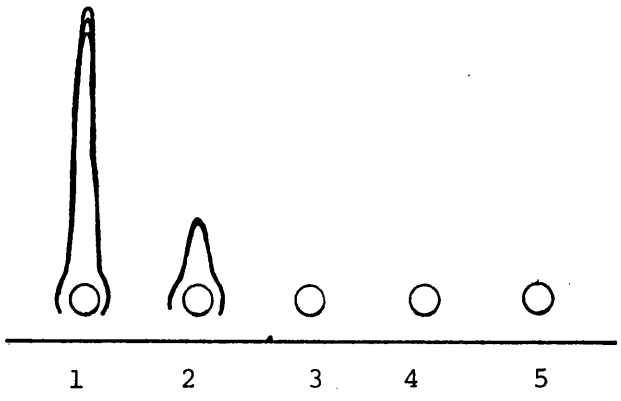


Figure 43. Rocket immunoelectrophoretic pattern obtained with Triton solubilized membrane fragments (1); Sephadex G-50 column fractions 10-22 (2); 23-27(3); 28-32(4); 33-50 (5) from Extract I. The agarose gel contained rabbit antibody to receptor-rich membrane fragments. (2%, v/v). Conditions: 16h. at 2V per cm.

6.5 Immunoelectrophoretic Analysis of Sephadex G-50 Column Fractions of Extract I

Rocket immunoelectrophoresis of column fractions of Extract I eluted from Sephadex G-50 was performed using rabbit antibody to membrane fractions and incorporating Triton X-100 solubilized membrane as a control. The precipitin pattern obtained, which shows precipitates for the detergent solubilized membranes and excluded peak (fractions 10-22) only is given in Figure 43. A previous analysis had also shown a precipitate for fractions 23 - 27, but this precipitate was not consistently repeatable.

When the excluded peak from this Sephadex G-50 elution was chromatographed on a column (1.5 x 60 cm) of Sephadex G-100, rocket precipitates were obtained for fractions eluted between 240 - 320 ml.

6.6 Immunoelectrophoretic Analysis of Extracts III; IV and IVa

Precipitates were obtained for Extracts III, IV and IVa upon rocket immunoelectrophoresis using rabbit antibody to membrane fragments and with Triton X-100 solubilized membrane fragments included as a control (Figure 44). When the pellets remaining after preparation of Extracts III, IV and IVa were analysed by rocket immunoelectrophoresis, together with the Extracts themselves, the precipitin pattern shown in Figure 45 was obtained.

6.7 Double Diffusion Assay using Lectins

Double diffusion assay incorporating lectins in serial dilution from neat to X16, was carried out with both Triton X-100 solubilized

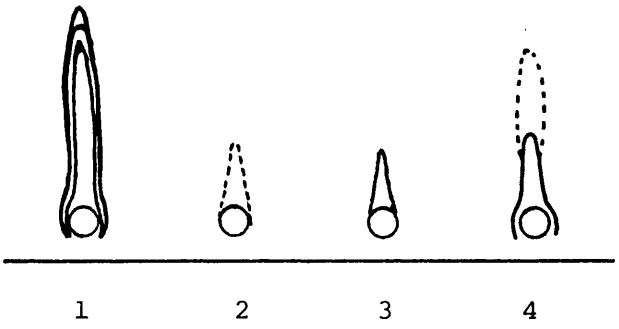


Figure 44. Immunoelectrophoretic analysis of Extracts III(2); IV (3) and IVa (4) using Triton solubilized membrane fragments (1) as a control. The agarose gel contained rabbit antibody (2% v/v) and the running conditions were 2V per cm. for 16h.

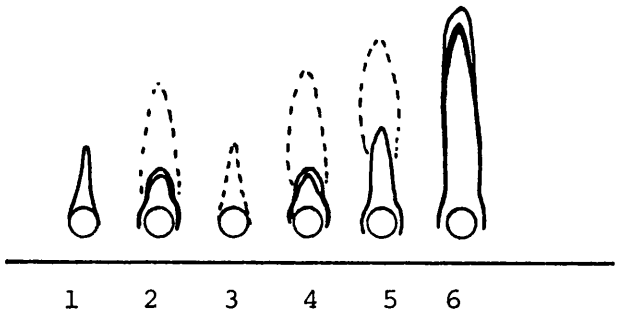


Figure 45. Immunoelectrophoretic analysis of Extracts IV (1); (III) (3); and IVa (5) together with the pellets remaining after Extract preparation. Pellet IV (2); Pellet III (4) pellet IVa (6). The gel contained rabbit antibody (2%, v/v) to electroplax membrane fragments.

membrane fragments and the excluded peak from Sephadex G-50 chromatography of Extract I. Under these conditions, no precipitates were obtained with either Concanavalin A or Maclura lectins.

6.8 Double Diffusion and Immunoelectrophoretic Analysis of Purified AChR using Rabbit Antibody to Membrane Fragments

Pure AChR from Torpedo marmorata kindly supplied by Dr. T. Barkas was assayed by double diffusion and 2-dimensional immunoelectrophoresis using rabbit antibody to membrane fragments as a control. No precipitates were obrained for the pure AChR with either assay, but the usual precipitates for the detergent solubilized control were demonstrated.

7. ACETYLCHOLINESTERASE ACTIVITY OF ELECTROPLAX MEMBRANE FRACTIONS

The following samples were assayed against suitable controls for the presence of acetylcholinesterase according to the method of Ellman et al. (1961).

(a) A suspension of membrane fragments in phosphate buffer

(b) Extract I

(c) Extract III

(d) Extract IV

(e) The pellet from the centrifugation step producing Extract IV.

(f) The excluded peak from Sephadex G-50 chromatography of Extract I.

The results are tabulated below.

Sample	Rate (μmoles per ml per min)	Protein (μg per ml)	Rate (μmoles per μg protein per ml)
(a)	105.08	5,000	21×10^{-3}
(b)	0.41	120	3.4×10^{-3}
(c)	1.20	130	9.1×10^{-3}
(d)	4.50	52	86.4×10^{-3}
(e)	4.53	40	113.3×10^{-3}
(f)	0.06	220	0.3×10^{-3}

Table 10. A comparison of the AChE activity of electroplax membrane fractions

8. CARBOHYDRATE ANALYSIS

For gas-liquid chromatographic analyses of individual hexoses and hexosamines in soluble carbohydrate containing fractions, aliquots were treated with 0.6 M HCl as described in the Methods section (p. 81).

8.1 Thin-Layer Chromatography (Stadler, 1976).

This technique was employed in order to try to separate the amino acids and monosaccharides obtained after acid hydrolysis of glycopeptides. Amino acid and monosaccharide standards were assayed together with acid hydrolyzed milk fat globule membrane glycopeptide. The separation achieved however using silica gel HR was not sufficient to warrant adopting the method routinely as an aid to gas-liquid chromatographic analysis.

8.2 Comparison of Derivatization Procedures

The conversion of sugar standards and those derived from biological samples to the corresponding trimethylsilyethers, met

with little success for two reasons: (1) this method involved derivatization of anomeric configurations which on analysis gave a complex pattern of peaks that were difficult to identify when native samples were investigated (Figure 46). (2) prolonged storage of such derivatives was of little value because of the ease with which they are hydrolyzed.

The conversion of sugars to their alditol acetate form was more successful as each sugar after conversion to the corresponding alditol reacts with acetic anhydride in its straight chain form, and so anomeric peaks are avoided (Oades, 1967). In addition such derivatives were very stable and consequently were easily stored. In order to establish that the technique was reliable two glycoproteins; bovine submaxillary mucin, and fetuin were processed and the results compared with those available in the literature. Standard sugars were derivatized and chromatographed in order to obtain Rf values for individual monosaccharides. Hydroxymethylmethylamine (Tris) was present in column fractions as a contaminant from the incubation buffer and could be converted to an alditol acetate form. Because it was present in all such fractions, and its position in the chromatogram was constant, it could be employed as an internal standard.

8.3 Maillard Compounds (Maillard, 1912).

Gas-liquid chromatographic analysis of acid-hydrolysed bovine serum albumin, which is known to contain no sugar residues, showed a complete absence of peaks on alditol acetate derivatization. When the experiment was repeated with the addition of glucose (1 mg), a number of small peaks were obtained, some of which ran

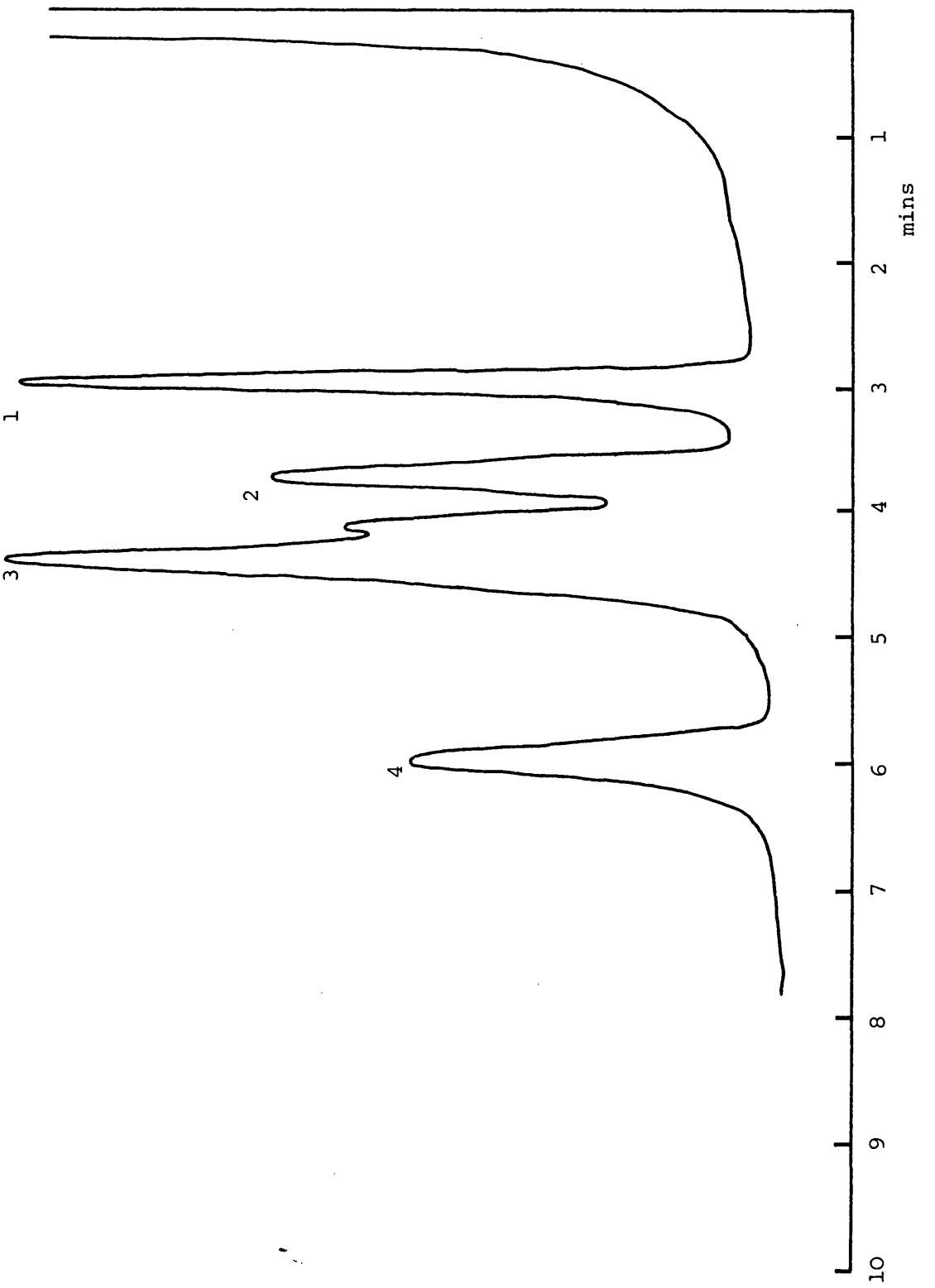


Figure 46. Diagram illustrating the relative position of TMS derivatives of mannose (1) glucose (2) glucosamine (3) and galactose (4). Conditions: temperature = 195°C packing: SE 30 Chromosorb (80-100 mesh)

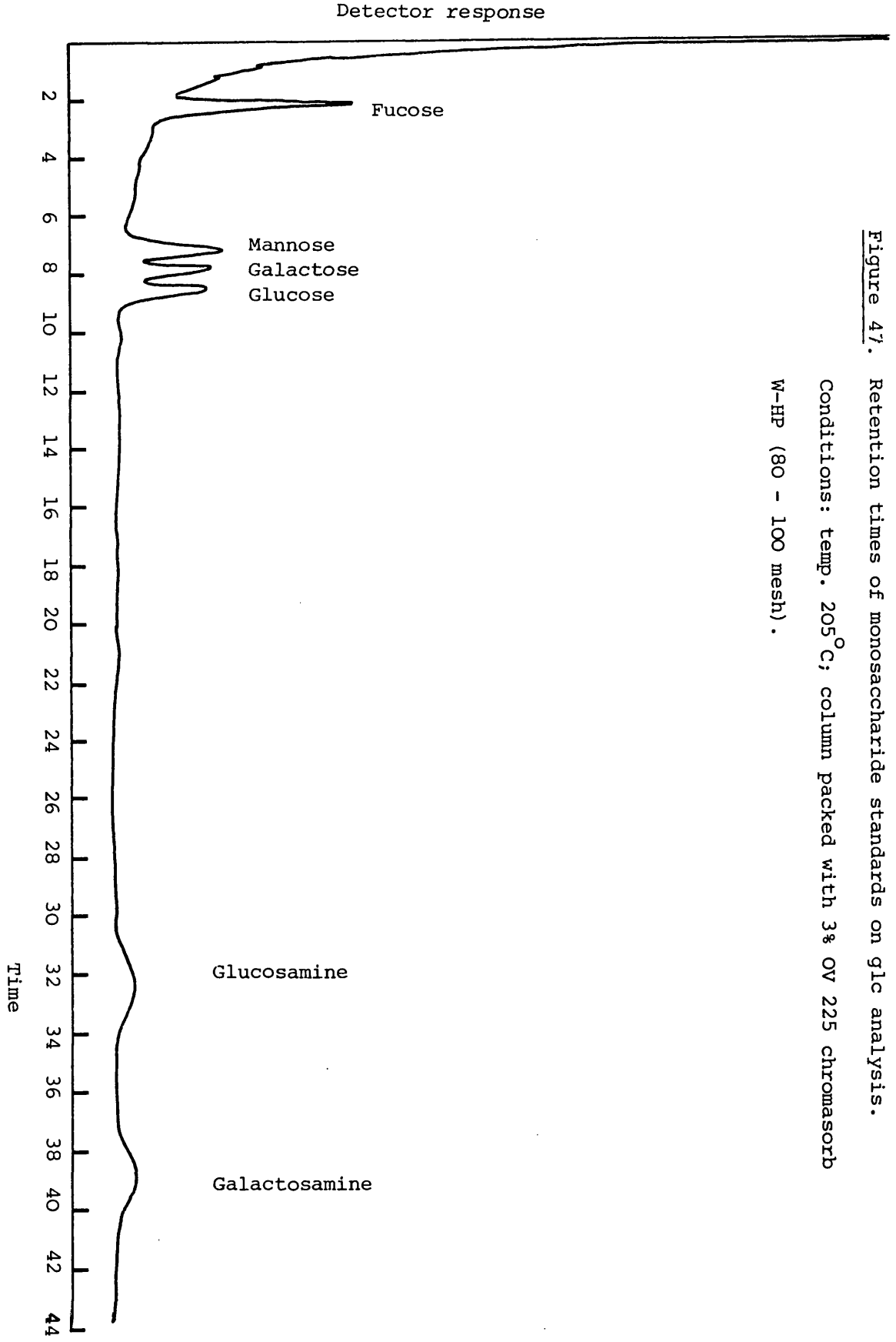


Figure 47. Retention times of monosaccharide standards on glc analysis.

Conditions: temp. 205°C; column packed with 3% OV 225 chromasorb W-HP (80 - 100 mesh).

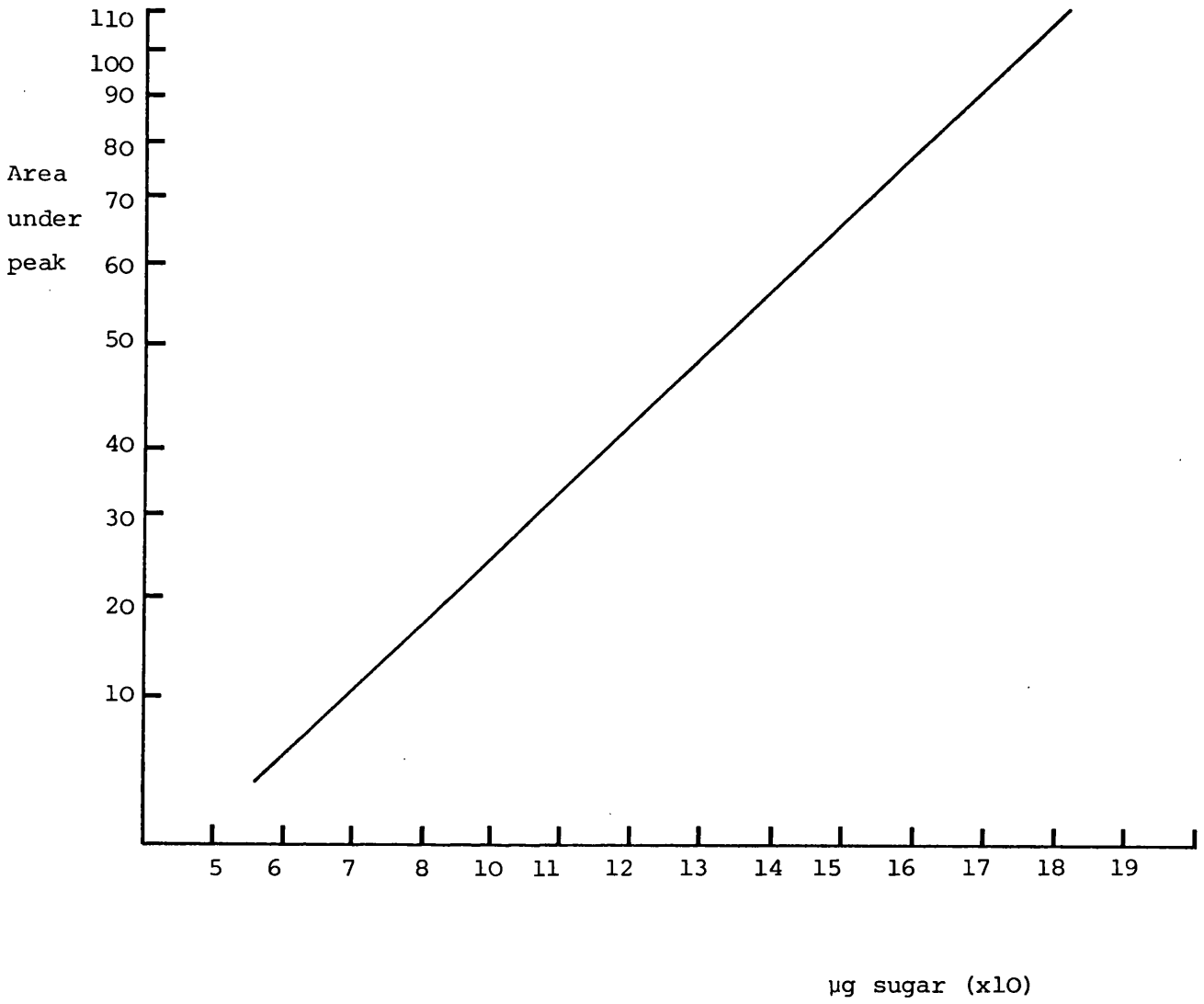


Figure 48. The linear relationship between sugar concentration and chromatogram peak area. Column packed with 3% OV 225 on chromabsorb W-HP (80-100 mesh) at 205°C.

in similar positions to native samples.

8.4 Retention Times and Rf Values of Monosaccharide Standards

These results are tabulated below. All values are expressed relative to hydroxymethylmethylamine (see also Figure 47).

Sugar Standard	Retention time (min)	Rf
Fucose	2.3	0.41
Mannose	7.2	1.29
Galactose	8.0	1.43
Glucose	8.6	1.54
Glucosamine	32.2	5.75
Galactosamine	39.6	7.07
Hydroxymethylmethylamine	5.6	1.00

Table 9. Monosaccharide standards - retention times and Rf values.

8.5 Preparation of a Standard Curve

Mannitol was used to demonstrate the linear relationship between sugar concentration and chromatogram peak area (Figure 48).

8.6 Analysis of Sephadex G-50 Column Fractions

The following sugars were found on glc analysis of fractions of Extract I eluted from Sephadex G-50 (Table 11).

Fraction one	Fraction two	Fraction three
(120-300 ml, eluted)	(300-400 ml eluted)	400-780 ml eluted)
Fucose	Fucose	Fucose
Mannose	Mannose (trace)	Mannose
Galactose	Galactose	Galactose
Glucose	Glucose	
Glucosamine		
Galactosamine		

Table 11. Monosaccharides identified from glc analysis of Extract I.

A sample of AChR (filtered through a 0.22 μ absolute filter) prepared and supplied by Dr. T. Barkas was also analysed for sugar content using gas-liquid chromatography (Table 12), (see also Figure 49).

Sample	Sugars found	Relative amounts
AChR (0.22μ membrane filtered)	Fucose	present
	Methylpentose	present
	Mannose	3 μg
	Galactose	1.5 μg
	Glucose	4 μg
	Glucosamine	present
	Galactosamine	present

The total carbohydrate found ≡ 35 to 50 μg per mg receptor protein

Table 12. Carbohydrate analysis of the AChR from Torpedo marmorata

A cholinergic receptor proteolipid sample supplied by Professor E.De Robertis was subjected to glc analysis together with Extracts III, IV and I(a) and a pronase sample (Table 13).

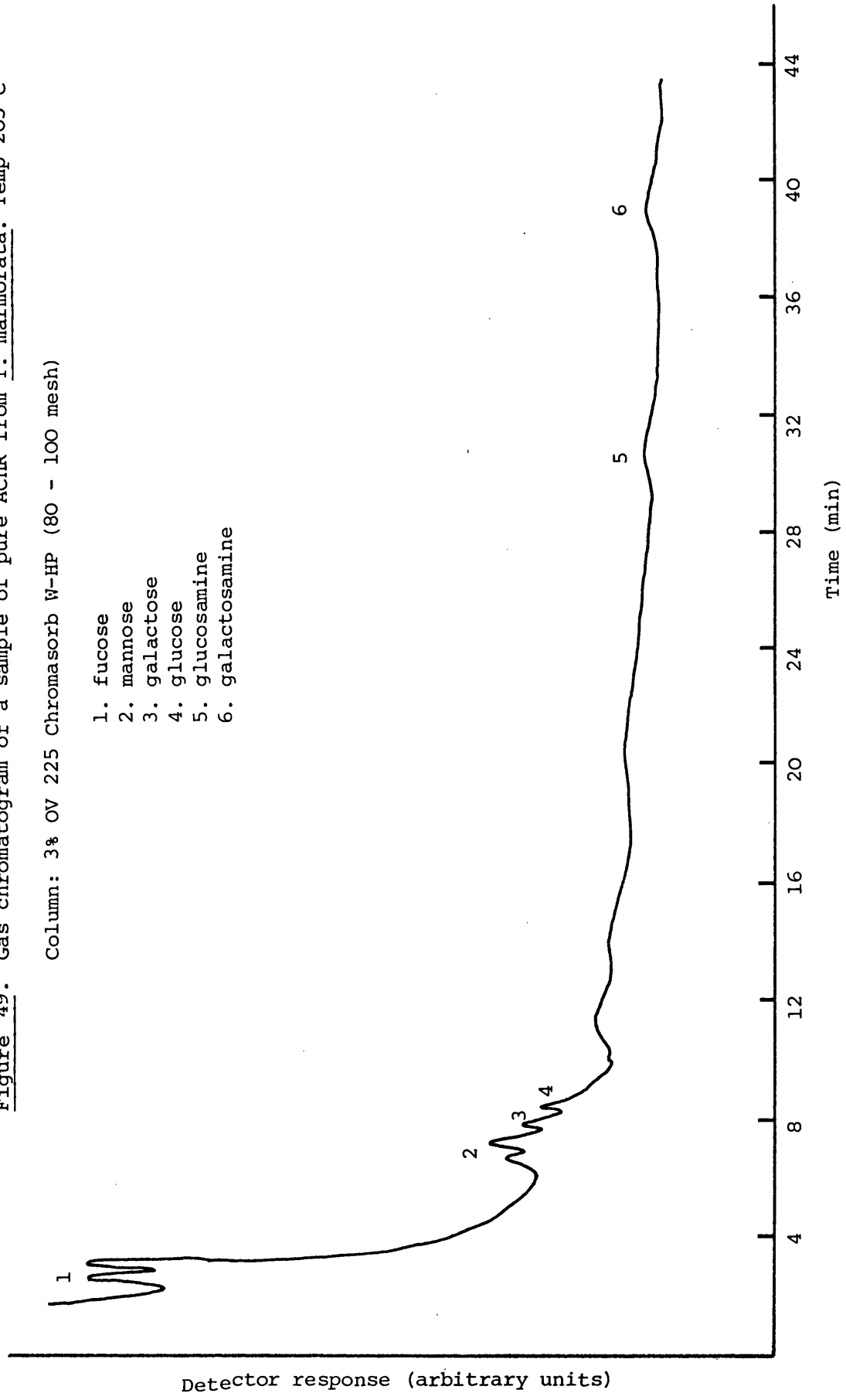
Sugar / Sample	Fucose	Methylpentose	Mannose	Glucose	Galactose	Glucosamine	Galactosamine
Proteolipid		o	o	o	o	o	o
Extract III	o		o	o	o	o	o
Extract IV	o		o		o	o	
Extract I(a)	o		o	o	o	o	
Pronase (Extract VI)			o	o			

Table 13. Carbohydrate contents from glc analysis.

Figure 49. Gas chromatogram of a sample of pure AChR from T. marmorata. Temp 205°C

Column: 3% OV 225 Chromasorb W-HP (80 - 100 mesh)

1. fucose
2. mannose
3. galactose
4. glucose
5. glucosamine
6. galactosamine



8.7 Sialic Acid Content of Electroplax Membrane Fractions

No traces of sialic acid were found either in proteolytic digests or the column fractions from a G-50 column elution of Extract I. In the assay a green rather than pink chromophore was given. No colour was given either by pronase or the tris incubation buffer alone.

9. TOXIN BINDING ACTIVITY OF ELECTROPLAX MEMBRANE FRAGMENTS

Assays for toxin binding activity were performed as described in the Methods section (p. 83). Tables 14 and 15 show the degree of toxin binding exhibited by fractions from the preparations of membrane fragments from Torpedo marmorata.

The tritiated α-bungarotoxin used for these determinations had a specific activity of 20 Ci per mMole at a concentration of 2.2. x 10^{-5}M. The stock toxin solution was diluted to give a specific activity of 60 nCi in 200 µl. This dilute toxin solution was incubated with membrane preparations as previously described. The counts per minute obtained for each sample were corrected for non-specific binding and background counts, before being compared with the total activity (TA) count obtained for dilute toxin solution alone.

Sample Volume	Corrected counts	Protein concentration	pmoles toxin bound per µg protein
1 µl	515cpm	0.027 µg	4.50
2	453	0.054	1.95
3	1136	0.081	3.25
4	1112	0.108	2.40
5	592	0.135	1.05
6	1061	0.162	1.55
7	1238	0.189	1.55
8	1861	0.216	2.00
9	1959	0.243	1.90
10	2009	0.270	1.75

TA = 12856

Table 14. Toxin binding by membrane fragments from Torpedo marmorata

The average figure for toxin binding from these figures is equivalent to 1.75 pmoles per µg protein. Considering the low specific activity of the tritiated toxin and the relative impurity of the membrane preparation, this figure would appear to be too high by at least a factor of ten. This was confirmed by using tritiated Naja toxin as shown in Table 15.

Sample volume	Corrected counts	Protein concentration	pmoles toxin bound per µg protein
1 µl	373	0.10 µg	0.30
2	662	0.20	0.27
3	699	0.30	0.19
4	1219	0.40	0.25
10	2726	1.00	0.44

TA = 6160

Table 15. Binding of Naja toxin by membrane fragments from Torpedo marmorata

The TA count was equivalent to 0.50 pmoles of Naja toxin. From the data shown, the average value for toxin binding is 0.27 pmoles per mg protein, which is equivalent to 270 nmoles per gram protein. A Triton X-100 solution extract of the 20,000 x g pellet obtained from Torpedo electric organ tissue was used in an assay with ^{125}I-labelled α bungarotoxin. The results are tabulated below

Dilution	Counts	Corrected counts	nmoles toxin bound per gram of protein
Neat	42484	39667	340
½	48234	45417	390
¼	52735	49918	430
⅛	55205	52388	450

blank = 2817

TA = 93096 = minimum 10 pmol per ml at ⅛ dilution. this is approximately equivalent to 800 pmol toxin per ml. Thus $\frac{\text{corrected counts}}{\text{Total Activity}} \times 800 = \text{nmol toxin bound per ml.}$

The protein concentration of the extract was 1 mg per ml

Table 16. Binding of ^{125}I-α bungarotoxin by Triton X-100 solubilized membrane fragments

10. RADIOIMMUNOASSAY (Barkas et al., 1978)

The binding of a radioactively labelled antigen to a fixed amount of antibody can be partially inhibited by the addition of unlabelled antigen, and the extent of this inhibition used as a measure of the unlabelled material added. The ratio of the free to bound radioactivity varies with the amount of unlabelled antigen added, enabling a calibration curve to be constructed.

In this assay iodinated AChR was mixed with Sephadex G-50 column eluted fractions of Extract I, and the unlabelled receptor was added followed by precipitation with antibody or Staphylococcus aureus. (Barkas et al, 1978, Wonnacott et al., 1980)

Table 17 lists the counts obtained together with the degree of inhibition shown by the column fractions.

Sample			Counts	% inhibition
Complete			11257	-
blank			358	-
fraction	(i)	(breakthrough peak)	2216	83
	(ii)	(tubes 23 to 28)	6283	46
	(iii)	(tubes 29 to 39)	3520	71
		(tubes 40 to 48)	6338	45
		(tubes 49 to 60)	5340	54

Table 17. Inhibition of AChR binding to rabbit antibody by Extract I.

The samples used in this radioimmunoassay contained Tris-HCl, $CaCl_2$ and NaN_3 and so they were dialyzed overnight against distilled water before being re-assayed (Table 18).

Sample		Counts	% inhibition
Complete		12391	-
blank		2247	-
fraction (i)	(diluted 1/3)	5097	72
	(diluted 1/6)	5501	68
	(diluted 1/12)	6021	63
	(diluted 1/24)	6660	57
	(diluted 1/48)	7294	50
fraction (ii)	(tubes 23 to 28)	8316	40
	(tubes 29 to 39)	12215	-
	(tubes 40 to 48)	10397	-
	(tubes 49 to 60)	11976	-

The dilutions of fraction (i) corresponded to 8 - 50 µg receptor per ml.

Table 18. Inhibition of AChR binding to rabbit antibody by Extract I following dialysis.

DISCUSSION

DISCUSSION

1. The results presented in this thesis were obtained with the intention of characterizing by using a number of techniques the proteins on the surface of membrane fragments prepared from the electric tissue of Torpedo marmorata.

Because the human disease myasthenia gravis and its animal model: experimental autoimmune myasthenia gravis (EAMG) involve an autoimmune response to antigenic determinants associated with the subsequent membrane of the neuromuscular junction (Aharonov, et al., 1975; Bender et al., 1975; Lindstrom et al., 1976; Fuchs et al., 1978; Harvey et al., 1978; Lindstrom, 1978), it was accordingly of interest to investigate the structural basis for the antigenicity shown by membrane fragments rich in AChR. The electric tissue from T. marmorata provides a particularly rich source of material for such investigations.

Membrane fragments were prepared according to the method of Sobel et al.(1977) and were characterised by measuring the binding of tritiated α bungarotoxin and also by polyacrylamide gel electrophoresis in sodium dodecyl sulphate solution. These membrane fragments which are probably derived from subsynaptic membranes of the electroplagues have been prepared by several investigators (Cohen et al., 1972; Flanagan et al., 1976; Sobel et al , 1977; Wennogle & Changeux, 1980) and have been found to show specific activities of up to 4500 nmoles toxin bound per gram of protein.

The fragments thus prepared bound tritiated $^{\alpha}$ bungarotoxin but the values obtained for specific activities (Table 14) were too high by approximately a factor of ten when compared to those obtained with Naja toxin (Table 15). This result is at variance with that to be expected using a toxin with a low specific activity, together with a membrane preparation where the receptor density would be relatively low.

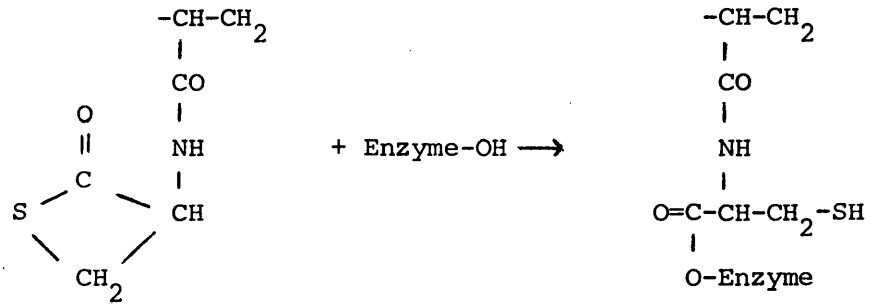
Fulpius (1976) has reviewed a number of tritiation methods specifically concerned with neurotoxins known to interact with AChRs. The first such derivative: mono ^{3}H acetyl-α-bungarotoxin described by Barnard et al. (1971) had the same affinity as the toxin for muscle receptors (Barnard et al., 1975) but had a specific activity too low for sensitive analysis.

In order to obtain radiolabelled toxins with a much higher specific activity, it has become common practice to employ iodination using ^{125}I. However, the iodination of proteins can potentially alter their biological activity and produce variable mixtures of labelled components exhibiting different properties. Coupled with this, the half life of ^{125}I is only 60 days compared to 12 years for tritium. A triton X-100 solubilized extract of membrane fragments bound approximately 450 nmols ^{125}I -α-bungarotoxin per gram of protein (Table 16), a figure which is much closer to that expected for preparations of this kind.

1.1 Proteolytic Digestion of Membrane Fragments

Membrane fragments were subjected to proteolytic degradation using pronase (E.C. 3.24.1.4 + 3.4.24.4), an enzyme mixture obtained from Streptomyces griseus which is known to contain endo, amino and carboxypeptidases together with a fraction that resembles bovine trypsin (Laskowski and Sealock, 1971). This enzyme preparation was preferred to trypsin in order that the proteolytic degradation of the membrane fragment surface would be as non-specific as possible. The incubation time was kept as short as possible, at 30 min, to avoid a complete degradation of all the membrane proteins present.

The use of soluble pronase was more successful in terms of the amount of glycopeptide material released, than pronase immobilized onto an inert adsorbent (Table 6) with only 81% of the protein released by soluble pronase being released at pH 7.6. The coupling of enzymes to Enzacryl is pH dependent and involves an attachment via hydroxyl groups from the enzyme itself. Thus different components of the pronase mixture will be bound by the Enzacryl at different pH values.



Barkas et al.(1978) have used pronase-Enzacryl with pure preparations of AChR. They demonstrated that over a 24h period, the antigenici ty of the preparation as measured by radioimmunoassay dropped to 1.2% of its original value. It may be that over an extended period of time, the amounts of polypeptide material released by soluble pronase and pronase coupled to Enzacryl at their respective pH optima would not be significantly different. Enzacryl immobilized pronase was originally employed to reduce the likelihood of contributing to the soluble glycopeptides obtained by pronase autodigestion. However, Arima et al.(1972) have shown that pronase contains less than 2% (by weight of carbohydrate and so will not contribute significant amounts of glycopeptide material to the digest.

The sonication of membrane fragments from Torpedo marmorata followed by the addition of pronase released approximately 50% of the available protein present, as assayed by Lowry's method. Wennogle & Changeux (1980) have claimed however that membrane fragments were resistant to both pronase and trypsin treatment unless sonication was performed in the presence of these enzymes. Their contention is that because the preparation of membrane fragments caused the formation of vesicles or microsacs in which the cytoplasmic side faces the inside of the structure, sonication was required in order to expose the receptor molecule to proteolysis. However, the membrane fragments used by the authors were stored at $4^{o}C$ in the presence of sodium azide for prolonged periods of time, and Wennogle & Changeux cite this condition as contributing to rendering the membrane subject to proteolysis.

It is possible of course that sonication only serves to remove and solubilize extrinsic proteins rather than to degrade integral proteins such as the AChR. Further experimentation employing PAGE would be required to classify the types of membrane proteins that are released following sonication.

The release of polypeptide material in the absence of pronase was achieved with Extracts III, IV and V although none of the conditions employed could match the performance of pronase in this regard (Table 7).

Extract III consisted of membrane fragments suspended in the incubation buffer used for pronase digestion, but minus the enzyme mixture, and was incubated at 37^{o}C for 30 min.

Patrick et al. (1975) have suggested that components of Mr < 48,000 are present in electric organ membrane fragment preparations only as a result of proteolytic digestion, and indeed results have been presented in this thesis which show that homogenates of Torpedo electric tissue possess a high degree of proteolytic activity which may in part be inhibited by the inclusion of both EDTA and PMSF during preparation (Table8). Michaelson et al. (1974) stated that they considered the possibility of proteolysis occurring during the isolation of membrane fragments unlikely, although they did incorporate both EDTA and PMSF into the buffers used for preparation. Their view was shared by Raftery et al. (1975) who considered that the subunits they obtained upon purification of the AChR were not proteolytically degraded from a larger entity. Raftery et al. do

conclude however, that some degree of proteolytic activity is present in electric tissue that is released upon "solubilization", and which if left unhibited will produce a marked alteration in the subunit pattern obtained. It is also interesting to note the comments of James (1978) who reported that aqueous preparations of PMSF rapidly become inactive. He has demonstrated that the half-lives of the inhibitor at 25°C were 110, 55 and 35 min at pH 7, 7.5 and 8 respectively. The presence of chelating agents such as EDTA and also variations in ionic strength had no effect on the rates of PMSF hydrolysis.

It is possible of course that the presence of endogenous proteases was responsible for the polypeptide release shown by Extract III. A second possibility is that the ionic strength (I = 0.1) of the buffer (tris-HCl containing 4 mM $CaCl_2$) was sufficient to remove any extrinsic proteins present on the membrane surface, and it was these that were detected rather than material from integral proteins.

Extract IV was essentially a sodium chloride extract of membrane fragment material and released approximately twice as much protein as Extract III. This may be explained by considering the contribution of both endogenous proteolysis and solubilization effected by an elevated ionic strength. Surface bound membtane proteins are readily released from the membrane structure in a water soluble form, often with full biological activity simply by altering the ionic strength of the suspending medium. Rosenberg & Guidotti (1969) have shown that a high proportion of the membrane proteins from human erythrocytes may be released into solution by

suspension in 1 M NaCl solution. Whether a selective, rather than a limited but generalized solubilization occurs is not clear, but evidence for the latter has been put forward by Schrager et al. (1972), although Mitchell & Hanaham (1966) demonstrated a 190-fold increase in the purification of AChE by extraction in 1.2M NaCl. It is important to bear in mind, however, the work of Mann (1972) which strongly suggests that the release of protein from cells during incubation in salt solutions may result from the activation of endogenous proteases. Thus the release of protein from membrane fragments by NaCl could be due to a combination of both ionic strength disruption of electrosatic binding and also of endogenous proteolysis which could account for the increase in protein release over and above that shown by Extract III. Chaotropic effects may be involved at very high ionic strength, but it is not likely that this is a contributory factor in this case.

Membrane fragments stirred with n-butanol at $0^{\circ}C$ produced Extract V. This organic solvent has been extensively used with erythrocyte ghosts, where virtually lipid-free proteins passed into the aqueous phase with lipids dissolved in the butanol phase (Maddy, 1966; Maddy et al., 1972; Rega et al., 1967; Zwaal & Van Deenen, 1968). Salton & Schorr in 1972 used butanol to extract the ATPase from Micrococcus lysodiekticus membranes, and Higashi et al. (1970) extracted hydrophobic proteins from Staphylococcus aureus.

This method of extraction did not approach the degree of

protein release shown by pronase, but was not significantly different from that shown by Extract III.

1.2 Sephadex G-50 Fractionation

Gel filtration of Extract I using Sephadex G-50 gave elution profiles which showed three main peaks, all of which were associated with hexose. The presence of some pronase autodigestion products is clearly seen in Figure 15; but the positions of the peaks do not coincide with those given by Extract I and although these products undoubtedly contribute to the fractions eluted from G-50, the amounts do not appear to be significant.

The elution profiles for Extracts III and IV are shown in Figures 16 and 17 and resemble the profile obtained for Extract I. The likely reasons for the release of protein under these conditions have been explored above and it is possible that a combination of proteolysis and the disruption of electrostatic binding contribute to the release of protein material in all three extracts, with just a shift of emphasis towards either effect depending upon the conditions employed.

The peaks eluted from G-50 are in the following molecular weight range: Peak A (excluded peak) > 30,000; B > 16,000; C > 12,000. The same profile was obtained regardless of the length of digestion time, but there was some variation in both the sizes and positions of the peaks.

The excluded peak (A) from Sephadex G-50 chromatography of

Extract I showed a precipitate on rocket immunoelectrophoresis using rabbit antibody to membrane fragments, and although peak B also showed a precipitate, it was not consistently repeatable and was probably an overlap from peak A.

When fractions corresponding to peak A from the Sephadex G-50 elution of Extract I were eluted from Sephadex G-100, an excluded peak was obtained which co-chromatographed with bovine serum albumin (M.W. 68,000 daltons), closely followed by a second peak a little further downfield. Rocket precipitates were obtained when fractions corresponding to this second peak were incorporated into an assay with rabbit antibody to membrane fragments (Figure 22). Thus the antigenic fragments cleaved from this membrane material had a Mr between 30,000 - 68,000 from gel filtration data. When the excluded peak A from Sephadex G-50 gel filtration was examined using SDS PAGE, five bands were obtained ranging from Mr 16,000 to 75,000 . The band exhibiting the lowest molecular weight also showed a positive PAS reaction, but the data from gel filtration and rocket immunoelectrophorsis suggests that this component is unlikely to cross react with rabbit antibody to membrane fragments. A number of peaks were shown downfield on G-100 elution of peak A which may correspond to the low M.W. material demonstrated by PAGE.

1.3 Polyacrylamide Gel Electrophoresis

Polyacrylamide gradient gel electrophoresis (PAGGE) was used to provide a comparison between fragments labelled with 4(N-maleimido)-benzyl ^{3}H trimethyl ammonium, generously supplied by Professor

J.P. Changeux of the Pasteur Institute, Paris, and unlabelled fragments prepared by the author.

In the presence of SDS the label was found to be concentrated in bands with approximate molecular weights of 40,000 and 60,000 daltons (Figure 23). The band of Mr 40,000 was found to be associated with the highest concentration of radioactive label. Corresponding bands with the same relative M.W. were also seen following PAGGE analysis of the unlabelled fragments. A number of investigators (Damle & Karlin, 1978; Reynolds & Karlin, 1978) have stated that as the ligand MBTA is specific for the ACh binding site, then the 40,000 dalton band which bound the highest concentration of radioactive ligand, must correspond to the subunit that carries the recognition site for acetylcholine.

When membrane fragments in Triton X-100 solution were subjected to SDS PAGE incorporating a stacking gel, 13 components were obtained with a Mr range between 12,000 to greater than 80,000 . Significant among these were bands 7, 8 and 9 which showed Mr of 66,000, 48,000 and 40,000 when compared to a calibration graph using standard proteins of known M.W. A preparation of membrane fragments solubilized in Triton X-100, which had been depleted of its receptor content by passage through a column of α-bungarotoxin coupled to Sepharose 4B was shown to contain both 66,000 and 48,000 bands, but the 40,000 Mr band had been replaced by a component with Mr 42,000 . The reported M.Ws. of the polypeptide chains comprising the receptor molecule differe depending upon the investigation and the species

of fish concerned. Reynolds & Karlin (1978) reported the presence of four polypeptides as did Hucho et al. (1978) and Wennogle & Changeux (1980). A comparison of the M.Ws. is shown below.

Chain	MW (Reynolds and Karlin, 1978)	MW (Hucho et al., 1978)	MW (Wennogle & Changeux, 1980)
α	39,000	42,000	40,000
β	48,000	48,000	43,000
γ	58,000	62,000	50,000
δ	64,000	68,000	66,000

The molecular weights quoted by Wennogle & Changeux (1980) alter upon degradation to give components of Mr 32,000, 35,000, 38,000 and 47,000. Thus the membrane fragments prepared for this investigation may have contained proteolytic degradation products in addition to the undegraded membrane proteins present.

SDS-PAGE was used to obtain Mr values for fractions of Extract I and also Extracts III and IV. The column fractions from Sephadex G-50 chromatography of Extract I show a number of components with Mr ranging from greater than 80,000 to less than 3,500. When pronase was examined under the same conditions, 6 bands ranging from Mr of 50,000 to less than 4,000. There appears to be little contamination of the column fractions by pronase components except for fractions 28-32, and 33-50 which could correspond to bands 4, 5 and 6 of the pronase sample B and 5 of the excluded peak from G-50 has a Mr of 16,000 which is very close to that shown by band 3 from the pronase sample. This band 5 was the only one to give a positive PAS reaction following PAGE.

The results obtained for the other extracts are inconclusive in that there appears to be little of significance apart from the fact that a number of components of diverse MW are present. The data do not make it clear whether or not the protein released in the absence of pronase was due to limited solubilization of extrinsic proteins, or a proteolytic degradation of integral proteins induced by the presence of high ionic strength. All that can be said with certainty is that the use of this technique has shown that the treatments used to produce the various Extracts examined, solubilize polypeptide components within a certain molecular weight range. Much more detailed work would be required, primarily to really identify what the actual method of solubilization was, and secondly to selectively solubilize components and compare these to the material solubilized by non-ionic detergents using SDS-PAGE analysis.

It has already been mentioned in the Introduction (p.38) that the binding of SDS to both glycoproteins and membrane proteins may be anomalous (Grefrath & Reynolds, 1974) and a linear relationship between mobility and $\log_{10}$ M.W. does not hold for such molecules. The empirical modification of this method reported by Segrest & Jackson (1973) has no firm theoretical foundation, and has been validated only for a limited number of glycoproteins. The answer to the problem of establishing true M.W. for such molecules probably lies in the technique of analytical centrifugation (Clarke, 1975; Tanford & Reynolds, 1976). Thus all the data presented here for PAGE analysis should be considered with respect to this information.

1.4 Membrane Fragments as Antigens

When membrane fragments emulsified in Freund's complete adjuvant were injected into rabbits, antibodies directed against many of the proteins comprising the membrane were induced, and were demonstrated both by double diffusion and immunoelectrophoretic assay using membrane fragments solubilized by Triton X-100 as the antigen. It has been shown by many investigators that non ionic detergents such as Triton X-100 dissolve membranes to a high degree (Gurd & Evans, 1974; Bjerrum & Lundahl, 1974; Bock et al., 1974; and do not affect the antigen: antibody ratio to any appreciable extent. (Crumpton & Parkhouse, 1972; Bjerrum & Lundahl, 1973; Nielson & Bjerrum, 1975). Optimum conditions for solubilization with Triton X-100 include a low ionic strength, alkaline pH and a final protein concentration of less than 5 mg per ml, all of which were complied with in the solubilization of the Torpedo membrane fragments. Any alteration to these parameters could result in detergent-membrane protein aggregates which would be difficult to break down, and would not elicit the desired antibody response upon immunization. The method employed for solubilization included a second extraction of the final membrane fragment pellet, because Bjerrum & Lundahl (1974) have shown that a repetition of the solubilization procedure often increases the yield of solubilized material.

Membrane proteins should, according to Gill et al. (1967) be good antigens because of their relatively high content of hydrophobic amino acids. In order that individual specific responses to antigen challenge were as far as possible eliminated,

four rabbits were immunized and their sera combined before the purification step. It is important to build up a pool of antibody when a reference pattern is established using immunoelectrophoresis, because the response to immunization in any one animal alters over a prolonged period of time, and the precipitation patterns obtained could differ significantly.

Immunization with a membrane fragment emulsion in Freund's adjuvant was preferred to a preparation solubilized in non-ionic detergent in order to avoid the induction of antibodies to the whole or parts of the detergent molecule. However, Bjerrum & Bog-Hansen (1976) have stated that immunization of material solubilized in non-ionic detergent does not give rise to the production of additional antibodies, although a higher titre is obtained possibly as a result of some adjuvant effect. The antiserum pool obtained was partially purified by $(NH_4)_2SO_4$ precipitation and desalted by gel filtration in order to increase the resolution of the precipitation patterns obtained, by diminishing the background staining often produced by unfractionated serum.

It is believed that each membrane protein gives a distinct immunoprecipitate in spite of the fact that Helenius & Simons (1972) have shown by gel filtration studies that the solubilization of such proteins with non-ionic detergents seems to produce large micelles. It may be that these large micelles are composed of more than one protein, but the electric field may somehow be responsible for disrupting the micellar structure to produce

single protein precipitates. The electrophoresis buffer at pH 8.6 confers a net negative charge on most proteins present in the system, and since the mean migration velocity of the antibodies is zero, in general it is the antigens only that migrate towards the anode. There may be proteins present in any system with a tendency to migrate towards the cathode, but by suitable adjustment of pH and ionic strength it is possible, according to Weeke (1973) and also to Bjerrum (1974) to induce most proteins to migrate towards the anode.

It has been shown by Bjerrum et al. (1975) that plasmin, a proteolytic enzyme present in serum is found in purified antibody preparations because the natural inhibitors normally present are removed by the purification procedure

This enzyme can give rise to artefacts in the precipitation patterns obtained because of proteolysis during the electrophoresis. Aprotium, which is a potent inhibitor of plasmin, was added to the rabbit antibody preparation immediately following the desalting step before the pool was frozen in aliquots, but despite this precaution the crossed immunoelectrophoresis patterns obtained exhibit the classic signs of proteolytic degradation: multiple peaks, split, slow and "flying" precipitates. It has already been mentioned that the homogenates of Torpedo electric tissue contain proteases and it is possible that these enzymes may be carried over to the detergent solubilized membrane fragment preparation where the degradation takes place.

Bjerrum and Bog-Hansen (1976) have shown that the presence of aprotinin, PMSF and soya-bean trypsin inhibitor were not effective in preventing the proteolytic degradation of human red cell membranes solubilized in non-ionic detergents. The Triton X-100 solubilized membrane fragments produced precipitation patterns showing four precipitates (rocket IE) and six precipitates (crossed IE). The inclusion of the receptor depleted membrane fragment sample showed that the rabbit serum contained antibodies directed against the many other membrane proteins present on the fragment surface, other than the AChR. It is interesting to note however that double diffusion assays using sheep anti-AChR serum gave precipitation lines for both the Triton solubilized membrane fragments and the receptor-depleted preparation. The particular receptor preparation used to immunize the sheep may have been of a relatively low purity with respect to the other membrane proteins present. Further work using crossed IE would be required to provide a comparison of the numbers and types as well as the positions of the immunogenic components present. A pure AChR preparation showed no cross-reaction with rabbit antibody to membrane fragments, but this is not unexpected because it is unlikely that a polyspecific antibody preparation induced by the immunization of rabbits with such material would be able to cross react with one highly purified membrane protein. The receptor density on these membrane fragments would be low compared to the quantities present of the many other membrane proteins.

Double diffusion assay using Extract I showed not only a

precipitate obtained with rabbit antibody to membrane fragments but also a reaction of identity with the precipitate given by the Triton solubilized membrane fragment preparation under the same conditions. Thus one of the components solubilized by Triton X-100 from membrane fragment is also released by treatment with pronase. The full significance of this result depends upon whether the component is an integral or just an extrinsic membrane protein. The latter, which is bound only by electrostatic forces would easily be released by detergent treatment and also by the relatively high ionic strength of the pronase incubation buffer. Conversely, if the component was partially embedded in the membrane bilayer the proteolytic activity would be required to remove a portion of that part of the molecule exposed at the surface and would correspond to one of the components released by detergent solubilization. The patterns obtained for both Extract I and Triton solubilized fragments certainly have some of the precipitates in common (see Figures 35 and 41). The proteolytic digests contained hexose, but no precipitates could be obtained by double diffusion of serial dilutions of lectins with either the excluded peak from Extract I or the detergent solubilized membrane fragments. This does not necessarily mean that the lectins used were not specific for any of the sugars present. Gas-liquid chromatography has demonstrated the presence of mannose in Extract I (Table 11) and one of thelectins used, Concanavalin A is known to bind the α-D-mannopyranoside configuration (Sharon & Lis, 1972; Lis & Sharon, 1973). It is possible however that the relative concentrations of reactants were not suitable for precipitation to occur or the pH of the

buffer used was too high, although this lectin has been used in crossed affino-immunoelectrophoresis by Schmidt-Ullrich et al. (1975) at pH 8.9. The absence of a reaction should not individually be taken as evidence that the relevant sugar is not present on the cell surface. If the gel has not been overloaded, those molecules present with binding sites specific for the lectin should react with it and form a precipitate. However, if the mixtures of proteins analyzed are still relatively impure, then clear cut examples of precipitates may not be obtained. The use of radio-labelled lectin especially with crossed immunoelectrophoresis incorporating intermediate gels would provide much more information concerning the sugars to be found on the Torpedo membrane fragment surface, and would possibly confirm the glc data obtained.

The excluded peak (A) from Sephadex G-50 chromatography of Extract I showed a precipitate on rocket immunoelectrophoresis, and although peaks also showed a precipitate, it was not consistently repeatable and was probably an overlap from peak A. As mentioned above, subsequent re-chromatography of peak A on G 100 gave a peak that exhibited precipitates on rocket IE.

The material released in the absence of pronase by treatments which led to Extracts III, IV and IVa showed precipitates following rocket IE but they were dissimilar to those obtained with both Extract I and the Triton X-100 solubilized preparation, and it may be seen by comparison of Figures 44 and 45 that very little antigenic material was actually lost from the pellets following these extractions.

1.5 Acetylcholinesterase Assay of Membrane Fragments

The enzyme AChE was shown to be present in a number of fractions of membrane fragments (Table 10), the highest specific activity being obtained from the pellet remaining following extraction of fragments with NaCl. The supernatant (Extract IV) showed the next highest specific activity which is surprising in view of the fact that AChE is routinely extracted from electric tissue using high concentrations of NaCl. This enzyme is a well known extrinsic protein associated with the electroplax membrane, and high ionic strength disruption of electrostatic bonding should be sufficient to remove the bulk of the protein. The third highest specific activity was exhibited by the membrane fragment preparation, followed by Extract III; Extract I and peak A from G-50 chromatography of Extract I.

Extract III exhibited three times the specific activity shown by Extract. I. This may indicate that while the buffer used for pronase treatment is conducive to AChE extraction, the presence of pronase significantly reduces the amount of enzyme present , by proteolysis. The excluded peak from Sephadex G-50 chromatography (peak A) of Extract I gave a specific activity equivalent to one tenth that of Extract I itself. It seems clear that AChE contributes very little to the composition of peak A despite the fact that it is present in significant quantities in membrane fragments and their extracts. Further data to support this conclusion could be obtained by assaying Extract I for AChE before and after extraction with NaCl solution followed by gel filtration on G-50 to recover peak A.

Purification of AChE from electric tissue and its incorporation into double diffusion assays and immunoelectrophoresis would determine the contribution made to immunogenicity.

Meunier et al.(1974) showed that AChE is removed from membrane fragments by washing with 0.8M NaCl, together with the other loosely bound proteins and that this treatment did not affect the subsequent binding of α toxins. Massoulie et al. (1969, 1970) reported that AChE from electric organ tissue occurs as three enzyme species of 8s, 14s and 18s all of which are converted by proteolysis to a 11s component. PAGE of AChE under reducing conditions gave components of M.W. 88,000 and 64,000. Dudai et al.(1972) have shown that two types of AChE may be purified from tryptic digests and from salt extracts of electric tissue.

1.6 Carbohydrate Analysis

The carbohydrate analysis carried out consisted of colourimetric determinations, thin layer chromatography (tlc) and gas-liquid chromatography (g l c).

Colourimetric methods were employed to estimate the amounts of hexose present in pronase and autoproteolysis digests and for the determination of sialic acid in column fractions of G-50 chromatography of Extract I. When the 2-thiobarbituric acid method (Warren, 1959; Aminoff, 1961) was employed for the determination of sialic acid a green chromophore was obtained rather than the usual pink colour. Warren (1959) has tested a

whole range of sugars, and with the exception of L-fucose none produced a chromophore or interfered with the colour formation. He has shown that L-fucose decreases the optical density in the method by 35%, and considers that this is due to the formation of acetaldehyde following periodate oxidation. The data obtained from g.l.c. analysis of peaks A, B and C (Extract I) together with Extracts III and IV show fucose to be present, and it is possible that the interference in the thiobarbituric acid assay may be due to this sugar. Hexoses were estimated by the cysteine: H_2SO_4 method (Dische and Danilchenko, 1967) alone, while hexoses and hexosamines were determined by gas-liquid chromatography. As discussed in the results section, two types of volatile derivatives are routinely used, viz. trimethylsily ethers and alditol acetates. The formation of TMS derivatives is rapid and it is possible to inject the reaction mixture directly into the chromatograph, but because of the formation of solvent equilibrium mixtures of pyranosidic, furanosidic and anomeric forms of the monosaccharides multiple peaks are obtained which can be confusing to interpret. The gas chromatography of TMS derivatives has been described by Sweeley et al. (1963).

Alditol acetates have now become very popular, being prepared by the borohydride reduction of monosaccharides produced by acid hydrolysis, followed by acetylation with acetic anhydride. The reduction of the carbonyl group during the preparation of alditol acetate derivatives eliminates the possibility of the formation of anomers or ring isomerization. Spiro (1972) and Laine et al. (1972) have written about the practicalities of

preparing alditol acetate derivatives and their estimation by gas-liquid chromatography. Ideally, the application of mass spectrometry is probably the best way to increase the reliability of the identification of chromatographically separated carbohydrates, and would give a valuable indication of the degree of purity of the individual preparations.

The ability to identify the sugars present in any membrane preparation depends to a very large extent on how efficiently individual monosaccharides are released by acid hydrolysis. The conditions for hydrolysis need to be carefully chosen because of the sensitivity of sugars to hot mineral acids. For example hexosamines are the most resistant to destruction by acid but they are less stable than most amino acids. Only 50% of amino sugars survive after heating at 105°C for 22h in 6 M HCl compared to 2 M HCl at 98°C for 6h. Glucosamine is slightly more sensitive in this regard than galactosamine. Sialic acids are rapidly destroyed on heating with dilute mineral acid for relatively short periods of time (0.01 M HCl at 100°C for 30 min.) Such destruction is not entirely due to the effects of acid since the exclusion of oxygen greatly reduces the destructive effect. In order to take advantage of this fact, nitrogen gas (O_2 free) was used to saturate the hydrolysate and fill the headspace of the vial before sealing, during the author's experiments. Mannose and galactose as non nitrogenous aldoses, occupy an intermediate position between these two extremes. For example 23% of mannose is destroyed by heating at 100°C for 5h. in 2 M HCl. Methanolysis causes less destruction of sugars than aqueous acid

hydrolysis and the resultant methyl glycosides are readily converted to volatile derivatives which may be determined by g l c

The hydrolysis system used to produce individual monosaccharides from Torpedo membrane fragment soluble glycopeptides was relatively mild (0.6 M HCl at 100°C for 16h. - see Methods section, p. 104). and in combination with N_2 saturation would not be expected to contribute significantly to sugar destruction. Thus the presence of fucose- normally a terminal sugar and like sialic acids readily hydrolyzed and destroyed - is seen in the G-50 column fractions of Extract I and also Extracts III and IV, as well as the pure AChR sample. The liquid phases OV-1 and SE-30 give comparable results from TMS derivatives when applied as a 3% coating on 80/100 or 100/120 mesh sialanized diatomaceous supports such as Chromasorb or Gas-Chrom Q. Alditol acetates are best chromatographed on 3% ECNSS-M on 100/120 mesh with nitrogen or helium as carrier gases. Helium is considered to give better resolution of alditol acetates with this particular liquid phase, however. Thin layer chromatograp hy of acid hydrolysates of glycopeptides was not successful under the conditions employed for the routine separation of amino acid and monosaccharide residues prior to g.l.c. analysis. The original method used radioactivity labelled compounds which were subsequently very much easier to detect following development of the Chromatogram than by using polychromatic sprays, and therefore higher yields of individual components would be obtained when eluted from their position in the chromatogram. It was demonstrated that Maillard compounds could be generated by processing a mixture of a monosaccharide and an aglycoprotein (BSA), but it is considered unlikely that under the routine

conditions employed such compounds contribute significantly to the traces obtained following g l c.

Because of its presence in the pronase incubation buffer, hydroxymethylmethylamine was used as an internal standard, the retention times of individual sugars being compared to that shown by this molecule to confirm their presence in the chromatogram.

The data for carbohydrate contents of the G-50 fractions of Extract I detailed inTable 11 show that fucose, mannose and galactose were common to all three fractions while glucose could be demonstrated as being present in the first two fractions, with the two amino sugars being associated only with the excluded peak A fraction. Extracts III, IV and Ia also contained these three hexoses, while glucose could only be demonstrated in Extracts III and Ia. Both amino sugars were present in Extract III but IV and Ia exhibited glucosamine only. When Extract VI (pronase enzyme mixture) was analyzed under the same conditions mannose and glucose only could be identified.

A sample of purified AChR supplied by Dr. T. Barkas was found to contain seven sugars (as detailed in Table 12) with the total carbohydrate content in the range 35 to 50 µg per mg protein (cf. Mattsson and Heilbronn, 1975). Six of these sugars were also demonstrated in a sample of receptor proteolipid supplied by Professor E. De Robertis. The presence of fucose could not be confirmed in this sample, although an unidentified methyl pentose was seen a little farther downfield on the chromatogram,

with respect to the expected position of fucose.

The presence of glucose in significant quantities in preparations derived from membranes is always contentious because of the ease with which standard preparative techniques can contaminate such preparations with the sugar. Eldefrawi and Eldefrawi (1973) reported that the source of carbohydrate in their purified AChR preparations was the Sepharose column used in affinity adsorption. It is interesting to note however, that this sugar is present not only in the relatively impure preparations of Extracts I, III and Ia, but is also exhibited by both the pure AChR and receptor proteolipid preparations.

The AChR has been characterized as a high M.W. glycoprotein by a number of investigators (Karlin et al., 1976; Raftery et al. 1976; Heidmann and Changeux, 1978; Aharonov et al., 1977) and the presence in preparations of AChR from a number of species, of mannose (Meunier et al., 1974; Michaelson et al., 1974; Heilbronn and Mattsson ,1975; Raftery et al., 1975), galactose and glucose (Michaelson et al., 1974; Heilbronn and Mattsson, 1975; Raftery et al., 1975) and amino sugars (Eldefrawi and Eldefrawi, 1973; Moore et al., 1974; Meunier et al., 1974; Raftery et al., 1975; Heilbronn and Mattsson, 1975). No other investigators have reported the presence of fucose or methyl pentose. Wonnacott et al.(1980) examined the effects of both fucosidases and galactosidase on the antigenicity of the AChR from Torpedo marmorata, and while not confirming or denying the presence of these sugars, showed that no loss could be attributed

to the effects of these glycosidases. Even following the loss of 70% of its Con A binding activity, the antigenicity of the receptor was unaffected.

1.7 Radioimmunoassay

Incorporation of G-50 column fractions of Extract I into a radioimmunoassay (RIA) with ^{125}I-AChR shows that peak A (fraction (i) - breakthrough peak) inhibits the binding of iodinated receptor by rabbit anti-AChR serum. This inhibition decreases in a linear fashion with increasing dilution (Table 18). Fraction (ii) (peak B) shows some degree of inhibition (40%) but this may not be significant because inevitably there will be some carry-over from peak A.

This assay has been used both by Barkas et al. (1978) to identify antigenic determinants on the affinity purified receptor, and the proteolipid described by De Robertis and his colleagues (La Torre et al., 1970) and also by Wonnacott et al., 1980 to demonstrate the inhibition of binding of radio-labelled AChR to Con A by $F(ab')_2$ and Fab fragments.

CONCLUSIONS

Membrane fragments prepared from Torpedo marmorata electroplax tissue exhibit surface acetylcholine receptor (AChR) activity with respect to the binding of snake neurotoxins and an affinity ligand. A number of the components present on the surface of the fragments are immunogenic and exhibit antigenic specificity. There are also indications that the AChR itself may be involved.

The precipitates obtained in immunochemical assays have been verified by performing reactions of identity and specific inhibition, using purified preparations of both antigen (AChR) and antibody (anti-AChR IgG).

The application of enzymic and detergent solubilization techniques has demonstrated that the surface components may be observed, defined and characterized in both a qualitative and quantitative manner.

Gas chromatographic analysis of soluble glycopeptides derived from the membrane surface, confirmed the presence of carbohydrate residues initially indicated using less sensitive chemical methods of analysis. The absence of sialic acid as a terminal sugar, reported by other investigators, was re-affirmed but the presence of fucose in the membrane fragment fractions examined may be significant in terms of replacing sialic acid in a terminal position.

The pure AChR molecule isolated from Torpedo marmorata is a glycoprotein.

Considerations for further work on this system should include an extension of the degradation of surface components by controlled enzymic treatment using those enzymes which do not exhibit such a broad substrate specificity as pronase, together with a range of glycosidases. The aim should be to utilize both enzymic and chemical methods of structural analysis to provide membrane derived glycopeptides which could contribute along with information obtained from immunological studies to the emergence of an overall

picture of the electroplax membrane surface. The further purification of such glycopeptides would provide a source of well defined and characterized carbohydrate molecule, the availability of which could facilitate the recognition of receptor structures on the membrane surface.

A supply of purified cell surface carbohydrates could be employed too, to extend the immunological studies already performed, by serving as haptens in the identification of immune sera raised against cell surface structures. This immune sera could then be used in the identification of the total surface structure, and pure glycopeptide or oligosaccharide molecules be immobilized onto a solid support to produce affinity columns for the specific isolation of membrane fragment components.

The identification, isolation and structural analysis of cell surface glycopeptides would provide much biologically significant information. The relative abundance of various carbohydrate structures indicating the size of the contribution made by glyoprotein molecules, to cell surface specificity.

More work needs to be performed to establish the numbers of discrete macromolecules present on the fragment surface, with particular reference to providing an accurate picture of the molecular weight range involved. This information would need to be correlated with the data obtained from carbohydrate structural studies in an attempt to clearly define the role(s) played by the major macromolecular components.

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