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**CONTROLLED DRUG DELIVERY BY MEANS OF
DRUG : IONIC POLYSACCHARIDE INTERACTIONS**

by

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B. Pharm., M. R. Pharm. S.

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ABSTRACT

The aim of the project was to investigate the potential of ionic polysaccharide/drug complexes as controlled release drug delivery systems.

Two highly purified alginates from *Laminaria hyperborea* and *Ascophyllum nodosum* were characterised in terms of molecular weight, polydispersity and M:G ratio using gel permeation chromatography (GPC), low-speed sedimentation in the analytical ultracentrifuge and GPC combined with multi-angle laser light-scattering.

Viscometric and nephelometric studies provided evidence that, above certain concentrations of propranolol, there was an interaction between propranolol and alginate in deionised water resulting in the formation of an insoluble complex, which dissociated in the presence of counter-ions, for example, sodium chloride.

Binding studies were undertaken using equilibrium dialysis in order to quantify this interaction in the presence and absence of sodium chloride. These indicated that there was a one-to-one stoichiometric relationship between propranolol and the carboxyl group on each uronic acid residue of the alginate and that negative co-operativity was occurring, such that the binding of one propranolol molecule to the alginate made it more difficult for subsequent propranolol molecules to bind. The possible *in-vacuo* three-dimensional structure of the molecular complex was modelled using computational molecular modelling techniques.

A freeze-dried complex of propranolol and alginate was prepared and characterised. *In vitro* investigations indicated that drug release from the complex (formulated as a suspension in deionised water or in isotonic glycerol solution) was delayed compared with release from a propranolol solution.

The release of propranolol from the propranolol/alginate complex was assessed *in vivo* using the anaesthetised rat as an animal model for nasal delivery. It was found that the rate of absorption of propranolol from the complex was much slower and was sustained over a greater period of time, compared with absorption from a propranolol solution. In addition, the bioavailability of the drug from the complex was comparable to that of the solution and to that of an intravenous dose carried out in rats by other workers (Hussain et al 1980b).

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To Johnny 'omes, with love. xx

GLOSSARY OF ABBREVIATIONS

∞	infinity
η	viscosity
<i>A. nodosum</i>	<i>Ascophyllum nodosum</i>
AUC	area under the plasma time curve
B	2nd virial coefficient
BN	batch number
c	concentration
d_v	volume equivalent diameter
ev	extravascular
F%	absolute bioavailability
F% _{rel}	relative bioavailability
G	guluronic acid
GPC	gel permeation chromatography
HPLC	high pressure liquid chromatography
I	ionic strength
IN	intranasal
IR	infra-red
IV	intravenous
K_d	dissociation constant
<i>L. hyperborea</i>	<i>Laminaria hyperborea</i>
L	litre
LSSE	low-speed sedimentation equilibrium
mL	millilitre

M	mannuronic acid
M_n	number-average molecular weight
M_w	weight-average molecular weight
M_z	z-average molecular weight
MALLS	multi-angle laser light-scattering
M:G ratio	mannuronic acid : guluronic acid ratio
NMR	nuclear magnetic resonance
UV	ultraviolet
V_0	void volume
V_t	total volume

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CHAPTER 1 INTRODUCTION

1.1 ALGINATES - GENERAL

1.1.1 Introduction

The alginates are naturally occurring polysaccharides which comprise alginic acid and its salts. Alginic acid was first discovered by the British chemist E.C.C. Stanford around 1880 (Stanford 1881). It is present in all species of the brown seaweed or Phaeophyceae (Cottrell and Kovacs 1977), and to a lesser extent in some species of the Corallinaceae or red algae (Okazaki et al 1982). A type of alginate has also been discovered in the extracellular mucilage of certain bacteria (Linker and Jones 1964, Gorin and Spencer 1966), for example *Azotobacter vinelandii*, and several species of *Pseudomonas* (Govan et al 1981, Linker and Jones 1966), although, at present, this is not used as an industrial source of the material.

In the Phaeophyceae, alginates are thought to have a skeletal function, giving strength, shape and flexibility to the plants. The Phaeophyceae include the species *Laminaria* (present along the coasts of Britain, Norway, France, North America, and Japan), *Ascophyllum* (Britain), and *Macrocystis* (U.S.A.). These abundant supplies are regularly harvested for the commercial production of alginates.

1.1.2 Commercial Processing

The alginate content of a seaweed can vary from 10% to 47% from one species to another and also between different parts of the same plant (Percival 1970).

In the brown seaweeds, alginate is usually present as the insoluble mixed salt (sodium, potassium, magnesium, calcium and sometimes strontium and barium) of alginic acid. The basic aim of

commercial processing is to convert this naturally occurring mixed salt of alginic acid into a soluble salt, for example, sodium alginate, which is relatively free from impurities.

In the commercial method of extraction (illustrated in Figure 1.1), seaweed is washed, then digested with alkali to dissolve the alginate, and filtered to remove insoluble material. Calcium chloride is added to the filtered solution to cause precipitation of calcium alginate. This is then acidified to precipitate alginic acid, which can then be converted to the desired salt by treating with the appropriate carbonate, oxide or hydroxide, and finally dried.

1.1.3 Structure

Originally, alginate was thought to be homopolymeric and to be composed of D-mannuronic acid (Nelson and Cretcher 1930). However, Fischer and Dörfel (1955), showed that hydrolysed fractions of laboratory-produced and commercial alginates contained L-guluronic acid as well as D-mannuronic acid. It is now well known that except for certain bacterial 'alginates' (Skjåk-Bræk et al 1986), alginates contain both L-guluronic acid and D-mannuronic acid.

In fact, algal alginates are linear block co-polymers of (1-4)-linked β -D-mannuronic acid (M) (Figure 1.2), and α -L-guluronic acid (G) (Figure 1.3) residues, in various proportions depending on the source (Haug and Larsen 1962). Studies have shown that the M and G residues are not arranged in a completely random manner, but that the monomers are arranged within the alginate molecule as a series of block structures (Haug et al 1967a). These blocks may be homopolymeric, that is, blocks of repeating D-mannuronic acid (M-blocks) or L-guluronic acid (G-blocks), or may be heteropolymeric, that is, blocks where the two acids alternate (MG-blocks).

Methods used to determine block structure in alginates have

Figure 1.1 Commercial Processing of Alginates

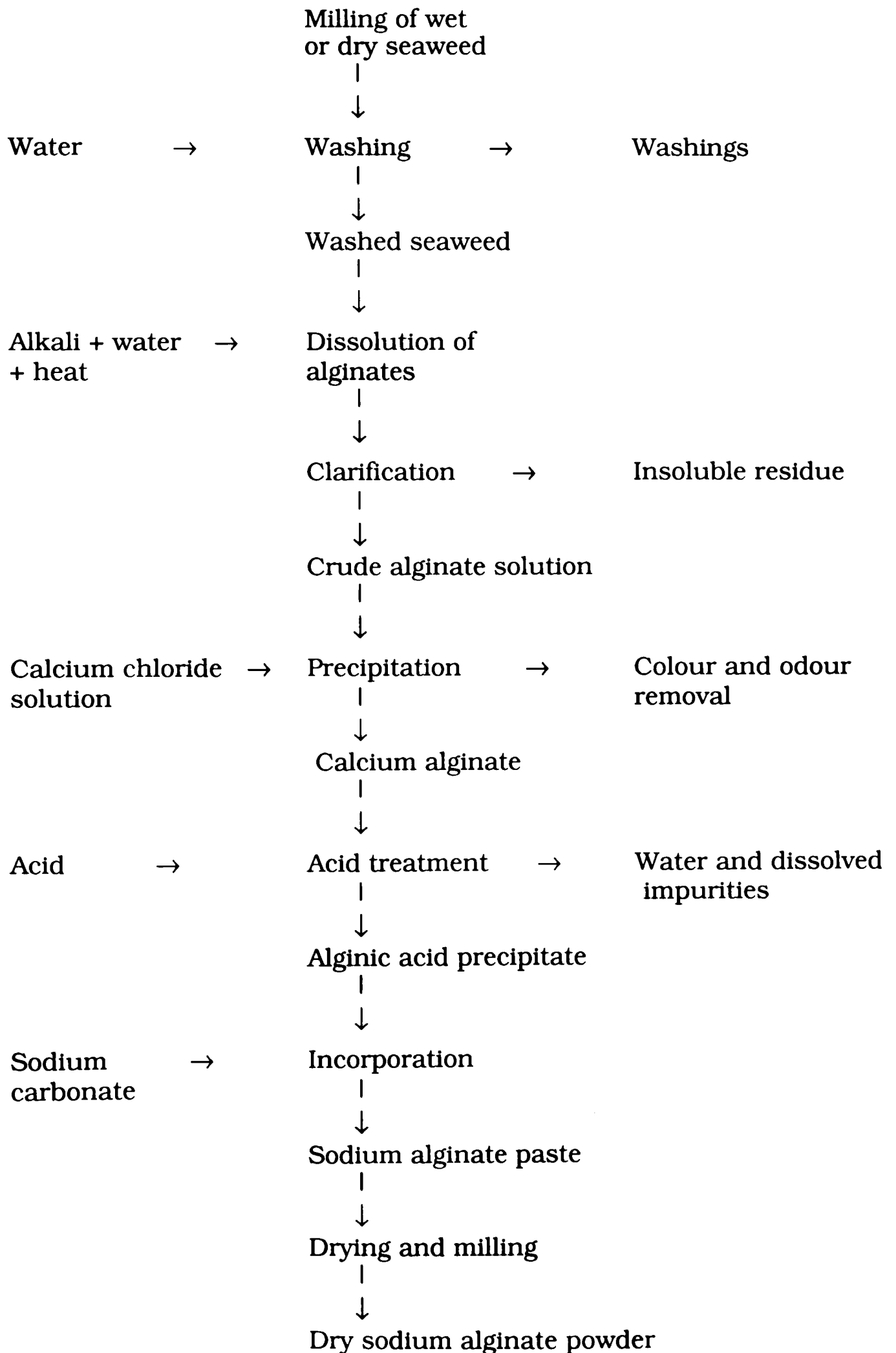


Figure 1.2 The structure of β -D-mannuronic acid
(4C_1 conformation)

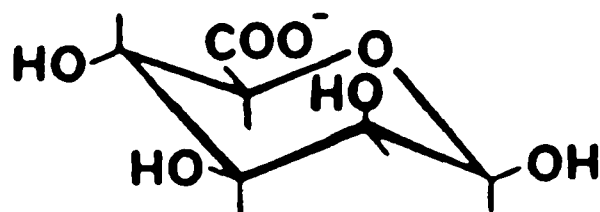
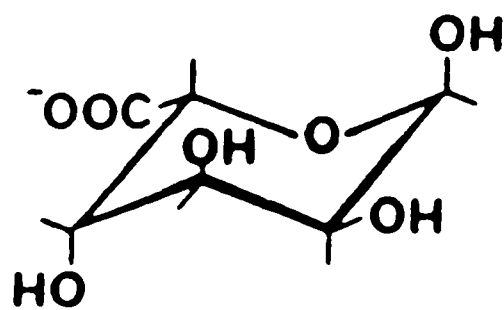


Figure 1.3 The structure of α -L-guluronic acid
(1C_4 conformation)



included: (i) IR spectroscopy (Mackie 1971); (ii) PMR spectroscopy following hydrolysis (Penman and Sanderson 1972); (iii) ^{13}C -NMR spectroscopy after enzymic hydrolysis (Boyd and Turvey 1978), and after mild acid hydrolysis (Grasdalen et al 1977, 1981); (iv) circular dichroism (Morris et al 1975); (v) ^1H NMR (Grasdalen et al 1979).

These and other investigations have demonstrated that alginates do not have any regular repeating copolymer structure (Painter et al 1968, Larsen et al 1970, Smidsrød and Whittington 1969). However, the most frequent structure within the alginate is blocks of M and blocks of G, connected by blocks of mixed M and G, of predominantly alternating structure.

1.1.4 Gelation Properties

In the presence of certain divalent and multivalent cations, for example Ca^{2+} , alginates form gels. The properties of these gels depends upon the mannuronic acid to guluronic acid ratio (M:G ratio) of the alginate. Alginates with a high guluronic acid content form strong brittle gels, whereas those with a high mannuronic acid content form weaker but more flexible gels. (Smidsrød and Haug 1972b, Penman and Sanderson 1972).

There are only minor differences in the structure of the two monomers and their affinity for divalent cations, thus the large difference seen in ion-binding by poly-guluronate and poly-mannuronate groups must have a macromolecular explanation:

In order that the bulky carboxyl group is in the energetically favoured equatorial position, the two uronic acids will adopt different preferred conformations. Thus, mannuronic acid adopts the $^4\text{C}_1$ chair conformation (Figure 1.2), and guluronic acid adopts the $^1\text{C}_4$ boat form (Figure 1.3). Evidence from X-ray diffraction studies supports this theory (Atkins et al 1971).

The orientation of the glycosidic bonds between the monomers will then affect the conformation of the polysaccharide

chain. Therefore, it has been predicted (Rees 1972), that regions in which poly-D- mannuronate predominates (M-M-M) will form an extended ribbon structure (Figure 1.4), similar to that of cellulose, whereas regions in which poly-L-guluronate predominates (G-G-G) will form a buckled chain (Figure 1.5). Further X-ray diffraction experiments by Atkins et al (1973a and 1973b), together with solution studies using $^1\text{H-NMR}$ (Penman and Sanderson 1972), and $^{13}\text{C-NMR}$ (Grasdalen et al 1977) provide evidence to confirm this prediction.

The mode of binding of cations by the various block structures within the alginate explains why the gels formed have different properties depending on the ratio of D-mannuronate to L-guluronate (M:G ratio). All the block structures are polyanionic and will form intermolecular bonds with divalent or multivalent cations, such as Ca^{2+} . However, poly-guluronate regions are also able to chelate the metal ion because of the spatial arrangement of the ring and hydroxyl oxygen atoms, thereby forming a much stronger type of interaction. Cavity-like sites are formed between adjacent G-units into which ions such as Ca^{2+} fit well (Figure 1.6). Thus, two or more chains may be joined together, side by side, by salt bridge formation between the G-blocks. This has been likened to the cross-section of an 'egg-box' (Grant et al 1973) as shown in Figure 1.7, where the Ca^{2+} ions are the 'eggs' within the 'egg-box'-like cross section of the alginate chains.

For this reason, when controlled amounts of calcium are added to alginate solutions, gels are formed. The calcium ions bind strongly to the G-blocks, but the formation of an insoluble precipitate is prevented by the presence of the M-blocks and the MG-blocks where the interaction with calcium ions is less. Since most alginates contain all three types of block structure (M, G, and MG), a classical gel structure can be formed where the G-blocks form the junction zones which are terminated by regions of M-blocks or MG-blocks. These remain dissolved and keep the

Figure 1.4 Chain conformation of poly- β -D-mannuronate ('extended ribbon')

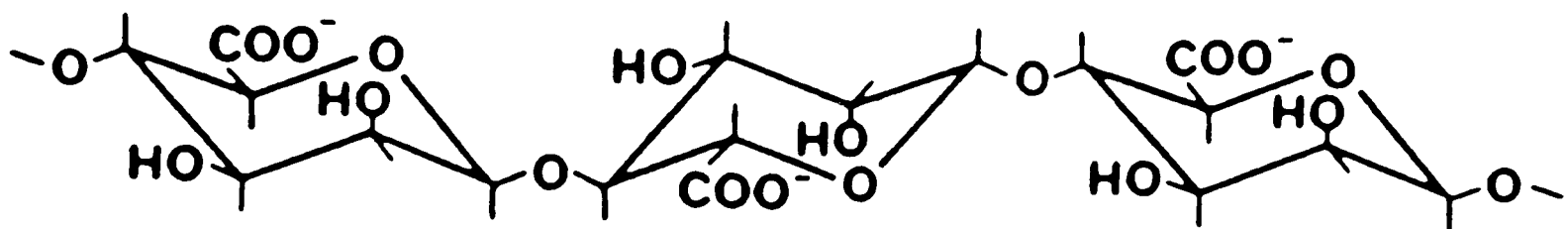


Figure 1.5 Chain conformation of poly- α -L-guluronate ('buckled chain')

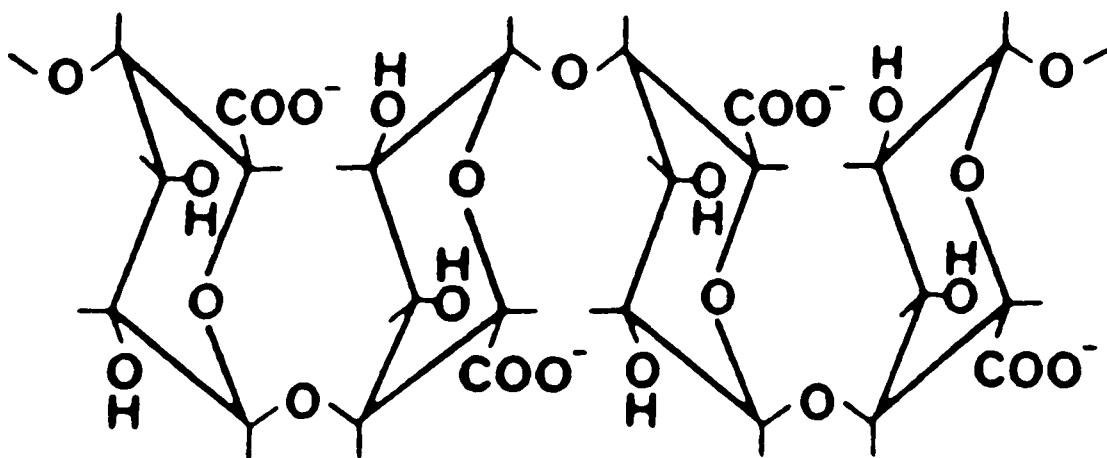


Figure 1.6 Calcium binding sites in G-blocks

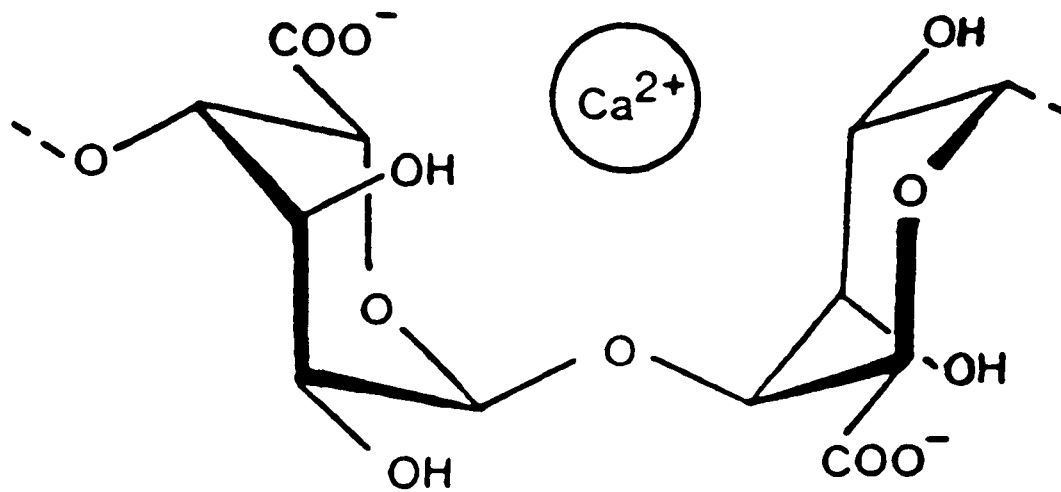
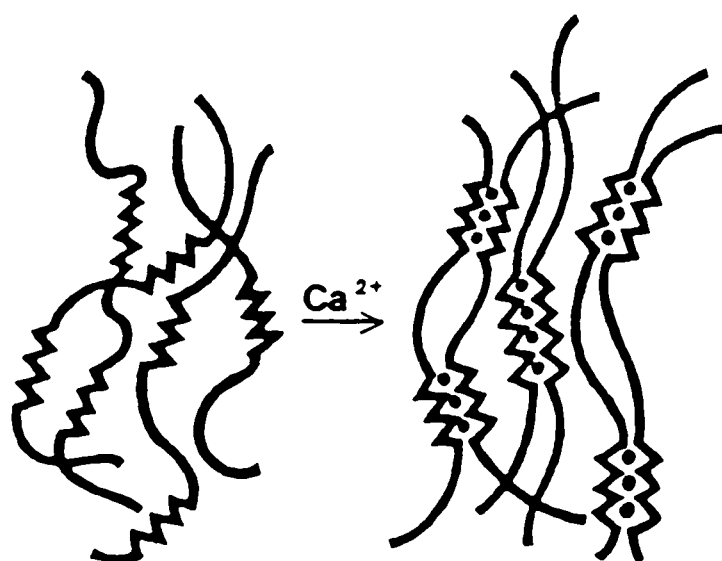


Figure 1.7 The 'egg-box' model



system in solution as a hydrated three-dimensional network, thereby forming a gel.

As already mentioned, alginate is generally believed to have a skeletal function in the brown algae. The M:G ratio of the alginate in different algae or in different tissues of the same algal plant reflects the relationship between alginate structure and its required function. For example, in *Laminaria hyperborea*, which grows in exposed coastal regions, the holdfast and stipe have a high guluronic acid content, giving strength and rigidity to these parts of the plant, whereas the blades, which float in the water, have a lower guluronic acid content, making them more flexible.

1.1.5 Toxicology

Many toxicological studies on alginates have been carried out. These have mainly taken the form of feeding trials, and have been summarised by McNeely and Kovacs (1975). They indicate that alginates are poorly absorbed by humans and that their consumption causes no significant harmful effects. Sodium alginate and propylene glycol alginate can be partially digested by colonic bacteria and thereby form part of the carbohydrate of the diet. However, the greater part remains undigested, and if eaten in sufficient quantities, may act as a bulk laxative (Berger et al 1953).

Alginates are natural products, and therefore contain impurities such as heavy metals, pyrogens and immunogenic materials, for example, proteins, complex carbohydrates and nucleotides (Paoletti et al 1987). The most common contaminants of alginates are phenolic compounds, which cause discolouration and catalyse oxidative-reductive-depolymerisation resulting in a decrease in the viscosity of the alginate.

For many applications of alginates, it is desirable that the above contaminants are removed, and this becomes a necessary requirement in applications such as transplantation (see section 1.2.2). All commercially available alginates are pretreated with

formaldehyde which converts the phenols into insoluble phenol-formaldehyde resin, but small amounts of phenolic compounds persist in all but the most highly purified.

1.1.6 Physical Properties

1.1.6.1 Stability

The stability of alginates in the solid state at moderate temperatures (less than 25°C) is excellent, but tends to decrease with increasing temperatures. For example, a sodium alginate with a degree of polymerisation of approximately 500, may be stored at 10°C to 20°C for three years with no observable change (McDowell 1977). If the alginate is more highly polymerised, hydrolytic degradation may occur to form alginates of lower molecular weight, and this becomes more serious at higher temperatures (above 50°C). Alginates are hydrophilic colloids and will absorb moisture from the atmosphere. The rate of depolymerisation increases in the presence of moisture, therefore they should be stored under cool, dry conditions.

1.1.6.2. Solubility

Alginic acid is virtually insoluble in cold water and only slightly soluble in hot water. This is because it is present largely in a crystalline state, giving it a very regular structure, in which there is ample opportunity for hydrogen bonding between the polymer chains. Therefore, the energy required to separate the molecular chains (the first step towards solution) is relatively high.

The presence of acid groups on each hexose unit of the alginic acid molecule means that it may be easily brought into solution by the formation of an ionised monovalent salt. For this reason, the salts of the alkali metals and ammonia are soluble in water. However, the salts of most of the divalent and polyvalent

metals have only limited solubility. For example, as discussed previously, calcium interacts with alginates to form the 'egg-box' structure (Grant et al 1973) illustrated in Figure 1.7. This is a stable structure, which holds together to form a gel in the presence of water. The larger strontium ion is more firmly bound than calcium ions thus the salt is insoluble in water, but the small magnesium ion is not able to stabilise the structure in the same way, so that magnesium alginate is soluble in water.

1.1.7 Solution Properties

1.1.7.1 Viscosity

The viscosity of alginate solutions is important for both theoretical and practical reasons. For example, the viscosity of extremely dilute solutions provides useful information about the size and shape of the alginate molecules. In many practical applications, such as when alginate is used at much higher concentrations as a thickening or suspending agent, it is the viscosity of the alginate solution which is critical.

Measurements of viscosity of most alginates are carried out in very dilute solutions, so that the interaction between the alginate molecules and the solvent can be studied, and complications arising from interactions between individual alginate molecules are minimised. These measurements are often expressed as the 'relative viscosity' or η_{rel} , which is the ratio of the viscosity of the solution η to the viscosity of the pure solvent η_0 at the same temperature. Another useful expression is the 'specific viscosity', η_{sp} which is defined as:

$$\eta_{sp} = \eta_{rel} - 1.$$

If the specific viscosity is divided by the concentration and a plot of η_{sp}/c versus c is extrapolated to zero concentration, this

ratio is known as the 'intrinsic viscosity' or $[\eta]$, which is a measure of the volume and shape of the space occupied by the molecules in solution (Florence and Attwood 1985).

The viscosity of alginate solutions depends mainly upon temperature, concentration, molecular weight of the alginate, pH, and the presence of monovalent and polyvalent cations. Each of these will be discussed briefly.

It is usual for any statement of viscosity to be accompanied by the temperature at which the measurement was made, because the viscosity of alginate solutions decreases with increasing temperature. This decrease is reversible provided that high solution temperatures are not maintained for long periods. Prolonged excessive temperatures lead to partial depolymerisation of the molecules, which results in a decrease in viscosity.

As the concentration of an alginate solution is increased, the viscosity increases almost exponentially. This is a result of entanglement and interaction between the alginate molecules which restricts the flow of the solution.

The relationship between the molecular weight and the viscosity of an alginate is more complex. Viscosity is determined largely by the length of molecules in solution, and thus molecules which are rigid in solution produce a higher viscosity than more flexible molecules of the same molecular weight. In general, alginates tend to be quite rigid molecules compared to other polysaccharides, and it has been found that this 'stiffness' is greatest in the G-blocks, followed by the M-blocks and lastly the MG-blocks (Smidsrød et al 1973). Alginates are also polydisperse materials, and the uncertainty about molecular weight distribution means that the relationship between the intrinsic viscosity of an alginate solution and its mean molecular weight can only be expressed as an approximation.

The pH of solution will also affect the viscosity, although between pH 5-10 the viscosity of alginate solutions is essentially

constant. Below pH 4.5 a significant viscosity increase occurs, due to the lower solubility of alginic acid, and below pH 3, insoluble alginic acid is precipitated. At a pH above 11.5, sodium alginates form gels. This is due to maximum ionisation of the alginate chains, resulting in mutual charge repulsion and a subsequent increase in the viscosity resulting in gel formation.

Polyvalent metal ions increase the viscosity of soluble alginate salts, and, as already mentioned, will cause gelation and/or precipitation if present in sufficient concentration. Small monovalent ions may cause a decrease in viscosity due to a reduction in charge on the alginate molecules resulting in their contraction in the solution.

1.1.7.2 Film-forming ability

Alginates possess excellent film-forming properties, and a wide range of soluble and insoluble films can be prepared. Soluble films can be formed by thin-layer drying of a solution. Extrusion of a soluble alginate into a precipitating solution (for example an acidic solution or a divalent or trivalent metal ion solution) produces an insoluble film. The films produced are tough, clear, flexible and resistant to grease, fats, oils and organic solvents, though they will transmit water vapour. When dry, the films are brittle, but they absorb moisture when in contact with normal atmospheres and become softer and more flexible. The film-forming effect of alginates is sufficient to hold together particles which have been wetted with an alginate solution and then dried. The alginate gradually decomposes on heating so that it is useful as a temporary binder in materials which must be fired, for example, ceramic glazes. The applications of alginates will be discussed further in the next section.

1.2. APPLICATIONS OF ALGINATES

1.2.1 General Applications

Alginates have an extremely wide range of applications which are intimately related to their basic physicochemical properties, and as already described, their lack of toxicity and relative stability at moderate temperatures. They produce viscous solutions at low concentrations, form soluble and insoluble films, and also form gels with many multivalent ions, especially calcium ions. These properties, together with their excellent stabilising and suspending properties have ensured that alginates have been extensively used in the food and drinks industry.

Alginic acid itself is relatively insoluble in water, therefore the most widely used compounds are the soluble salt sodium alginate, or the ester propylene glycol alginate.

The uses of alginates in the food industry have been widely reviewed (McDowell 1960, Glicksman 1969). They have been used in the food and drinks industry as thickening, suspending, stabilising, emulsifying, gel-producing and film-forming agents, which have the added advantage that they do not inhibit or mask flavours. A brief summary of the applications of certain alginates in the food industry is provided in Table 1.1.

Alginates are also used for a variety of purposes in industry. They have a wide range of applications in the textile, welding and paper industries. Table 1.2 outlines some of these applications.

A future application of alginates is in agriculture and forestry. At present, the widespread use of herbicides and other pest-controlling chemicals has led to the excessive introduction of toxic substances into the environment. Pfister et al (1986) investigated calcium alginate gel formulations for the controlled release of herbicides, and found that release of the herbicides could be maintained over several months, thereby reducing the need for

Table 1.1 Some Applications of Alginates in the Food Industry

<u>Property of Alginate</u>	<u>Food Product</u>	<u>Application of Alginate</u>
Gelation	Dessert gels	Produces clear, firm, quick-setting gels with hot or cold water.
	Pie fillings	Develops soft gel body with broad temperature tolerance.
Emulsifying	Salad dressings	Emulsifies, stabilises and modifies flow properties in pourable dressings.
	Meat and flavour sauces	Emulsifies oils and suspends solids.
Stabilising	Beer	Maintains beer foam under adverse conditions.
	Sauces and gravies	Thickens and stabilises.
Water-holding	Dry mixes	Absorbs water or milk quickly upon reconstitution.
	Syrups	Suspends solids, controls pouring consistency, provides body.

Table 1.2 Some Industrial Applications of Alginates

<u>Property of Alginate</u>	<u>Product</u>	<u>Application of Alginate</u>
Water-holding	Adhesives	Controls penetration to improve adhesion and application.
Gelling	Air freshener gel	Firm stable gels are produced.
	Toys	Safe, non-toxic materials are made for putty-like modelling materials.
Emulsifying	Polishes	Emulsifies oils and suspends solids.
Binding	Ceramics	Imparts plasticity and suspends solids.
Filming	Paper sizing	Improves surface properties, ink acceptance and smoothness.

repeated applications of excess volumes of chemicals. This, together with the fact that the polymer matrix is biodegradable means that the replacement of conventional pesticide formulations with controlled release systems could have great advantages both ecologically and economically.

1.2.2 Pharmaceutical and Medical Applications

In the pharmaceutical industry, alginates are used not only as clinically active materials, for example in wound dressings (section 1.2.2.2), but also as excipients in many drug formulations.

As excipients, alginates may be used, for example, as swelling agents, stabilisers and suspending agents. The great variety of alginates commercially available means that important factors such as viscosity and thermostability may be selected for each individual product.

In the past, alginates were a popular choice as disintegrants in tablet production, since alginic acid swells in contact with water without dissolving. However, they have largely been replaced by the semi-synthetic starch and cellulose derivatives which provide improved control of disintegration.

The film-forming properties of alginates are exploited in their use as enteric coats for tablets, since the films are insoluble in the presence of acid, but dissolve rapidly in the alkaline conditions of the lower gastrointestinal tract to effect drug release.

This insolubility in acid also means that alginate is an effective anti-ulcer agent as it forms a protective film on the stomach lining. It has buffering capacities which make it useful as an antacid in the treatment of dyspepsia and gastric reflux. Pharmaceutical preparations on the market at present which contain alginates include 'Gaviscon', 'Gastrocote', 'Algicon', 'Gastron' and 'Topal', which are available in tablet and/or liquid form.

'Gaviscon' contains alginate and a carbonate or bicarbonate, which releases carbon dioxide gas on contact with gastric acid.

Bubbles of gas become entrapped in the alginate gel network, which consequently floats on the stomach contents as a viscous layer. Two possible modes of action have been suggested (Washington et al 1986). Firstly, a physical barrier or 'raft' is formed on top of the stomach contents, thereby preventing contact of stomach acid with the sensitive epithelium of the oesophagus. As a secondary mechanism, if the raft is ruptured, only neutral raft material is refluxed into the oesophagus instead of the acidic gastric contents which usually cause the symptoms of 'heartburn'.

Sodium alginate tablets have been administered in nuclear accidents in order to bind radioactive strontium which may be ingested with food (Einig and Knoll 1991). An insoluble salt is formed (section 1.1.6.2) which is then excreted.

Another useful application of alginates is that entrapment of living cells (for example, from bacteria, yeasts, plants and animals) in calcium alginate gels or in microcapsules of certain alginate salts can be achieved under mild conditions which cause no damage to the cells. It has been suggested that alginate gels containing pancreas cells could be used for the transplantation of insulin-producing tissue, possibly without an immune response (Skjåk-Bræk et al 1989), and this will obviously have important consequences for future medical research into implantation and transplantation.

In dentistry, alginates are used extensively as impression materials and dental adhesives. In a recent patent, Keith (1990) has described a further use for alginates in dentistry. Alginate was combined with chlorhexidine, a divalent cationic antiseptic, to form a precipitate. The author suggests that the powdered precipitate is suitable for inclusion in a professional dentifrice or in a consumer toothpaste, and has the advantage that it avoids the bad taste and staining of tooth enamel that are normally a problem with chlorhexidine-containing formulations.

1.2.2.1 Agents for Controlled Release of Drugs

As well as their traditional role in the pharmaceutical industry

as described above, alginates, together with other natural and synthetic polymers, are now being exploited for their use as agents to provide controlled delivery of drugs, and have applications in most routes of administration. For example, sustained oral drug delivery can be achieved by incorporating drug into a hydrophilic matrix tablet or into a gel formulation. Transdermal drug delivery and depot injections can be controlled by the choice of a suitable polymer. Also, the potential use of alginates as implantation materials has been mentioned above.

The use of alginates and other polysaccharides in hydrophilic matrix tablets is well documented (Melia 1991). At a simple level, a hydrophilic matrix system consists of a compressed mix of a drug with a viscous polymer which swells in water. In practice, many other excipients are added to improve the properties of the tablet. When the tablet comes into contact with body fluids, a thin gel forms on its surface from which the active drug is released by a combination of diffusion through the gel and erosion of the gel surface. The tablet core stays dry and provides a reservoir both of active drug and of polymer to replace the gel layer as it is dissolved and eroded in the gastrointestinal tract. A very successful hydrophilic matrix system containing alginate and verapamil has become one of the best selling antihypertensive drugs worldwide (Einig and Knoll 1991).

Alginate gels have also been studied as oral drug delivery systems. For example, Stockwell and Davis (1986) formulated gels containing sodium alginate, calcium ions, sodium bicarbonate, and a drug, resulting in a floating gel system as described for 'Gaviscon' in section 1.2.2. Sustained release was achieved for the anionic drug sodium salicylate, and to a greater extent for the cationic drug chlorpheniramine maleate, an effect suggested to be a result of electrostatic interactions between the positively-charged drug and the negatively-charged alginate.

Calcium alginate beads have also been investigated for controlled release of drugs. The use of alginates to immobilise living cells has been discussed in section 1.2.2. This research led to studies

investigating the use of calcium alginate beads as a sustained release dosage form. Preparation of the beads is relatively simple. As a general method, a sodium alginate solution is prepared, into which drug is dispersed. This is then forced drop by drop through capillary tubing into calcium chloride solution, where insoluble calcium alginate beads are formed. These are washed and then dried at room temperature. For example, Badwan and co-workers (1985) prepared alginate beads impregnated with sulphamethoxazole and reported sustained release of the drug. Bodmeier and Paeratakul (1988) formed alginate beads using poorly soluble drugs such as ibuprofen, indomethacin and tolbutamide, which reportedly did not disintegrate in acidic conditions, but released drug in intestinal fluids.

1.2.2.2 Wound Management

Alginates have gained great importance in the field of wound management. In 1962, it was observed by Winter that epithelisation of wounds beneath polythene films progressed at approximately twice the rate occurring under a dry scab (Winter 1962). This led to the philosophy of 'moist wound healing'. However, the occlusion of wounds with films was not immediately employed in wound management, due to the tendency for bacterial growth below the films.

The development of materials such as 'Op-Site' (almost ten years later) which allowed the diffusion of air and water vapour which seemed to inhibit bacterial growth, led to the widespread use of film dressings. These original films had the disadvantage that, although they retained wound exudate in contact with the wound, thereby promoting moist healing, the exudate eventually became difficult to contain. Thus, hydrophilic foams, hydrogels and hydrocolloids which could absorb the exudate without affecting wound healing were developed.

Originally, two products based on calcium alginate fibre were launched: 'Sorbsan' and 'Kaltostat'. Since then, these products have

been developed further, and 'Sorbsan SA' and 'Kaltoclude' are now available. According to the British National Formulary (1992), these products act as 'occlusive or semi-occlusive dressings which adhere to dry skin and interact with moisture in the wound to form a gel'. They are employed as treatments for exuding lesions, for example, pressure sores, burns and graft-donor sites.

Another wound product based on alginate is 'Stop Hemo' which, as its name suggests, is used to arrest bleeding. When employed as a haemostatic agent, sodium alginate interacts with blood calcium to form cross-linked calcium alginate which seals the lesions and stops bleeding. It has been shown that alginate dressings can eliminate the need for blood transfusions following blood loss in certain types of surgery (Blair et al 1990).

As wound healing agents, alginate dressings are used in preference to traditional products based on cotton, lint or gauze because they can be easily changed with no discomfort to the patient and relatively little damage to the wound itself. This is because, in use, the calcium alginate fibres slowly release calcium ions in exchange for sodium ions from the wound exudate, which liquefies the fibres. Therefore, dressings may be left in place for up to seven days, but, at any stage, washing with a sterile saline solution will liquefy and dissolve the dressing and with it the absorbed wound exudate.

1.3 INTERACTIONS OF ALGINATES

1.3.1 General

Hydrocolloids such as alginates are long chain, high molecular weight polymers which dissolve or disperse in water (Burger 1982), and are often used to increase the viscosity of the medium in which they are dissolved. However, the behaviour of a hydrocolloid molecule in aqueous solution depends upon the interactions which occur between:

- (a) hydrocolloid and solvent molecules;
- (b) individual hydrocolloid molecules;
- (c) hydrocolloid and other solute molecules in the system.

Also, there may be indirect effects, for example, competition between hydrocolloid and other solute molecules for available water molecules, thereby changing the degree of hydration of the hydrocolloid.

In very dilute solutions, the hydrocolloid-solvent interactions tend to be maximised and the intermolecular hydrocolloid-hydrocolloid interactions are minimised, resulting in maximum extension of the hydrocolloid molecule and therefore a high intrinsic viscosity (section 1.1.7.1). Increasing the concentration of hydrocolloid may lead to intermolecular interactions where the polymer coils begin to overlap (Mitchell 1979). A phenomenon known as 'entanglement coupling' occurs in concentrated solutions, such that the solution behaves like a crosslinked network (where the crosslinks have short lifetimes), thereby causing a marked increase in the viscosity of the solution (section 1.1.7.1).

Hydrophilic polysaccharide polymers may be classified into non-ionic types (for example, cellulose ethers) or ionic types (for example, alginates, carrageenans and xanthan gum) which have carboxyl or sulphate side groups on the polysaccharide chains. Intermolecular interactions of polyanionic hydrocolloids, such as sodium alginate, may show differences compared with intermolecular interactions of non-ionic hydrocolloids. For example, the presence of charge on the molecule may either inhibit or promote intermolecular interactions. In addition, cations, whether unintentionally present or deliberately introduced, may have a specific role in the formation of intermolecular interactions, for example, as in the 'egg-box' model for the gelation of alginates in the presence of calcium ions.

1.3.2 With Metal Ions

The interaction of polyuronates such as alginate with calcium ions has been discussed previously and has been described by Grant (1973) using the 'egg-box' model in which the interstices of the G-blocks of the alginate chains are filled with Ca^{2+} ions. For biological and biotechnological reasons, this system has received the greatest attention, but other divalent ions, for example Zn^{2+} , Cu^{2+} and Cd^{2+} , and some trivalent ions such as Bi^{3+} and Cr^{3+} have also been investigated (Césaro et al 1988).

Other divalent cations may be incorporated within junctions in a similar manner to calcium ions, but there are noticeable differences in the strength of binding, which have been widely investigated (Haug and Smidsrød 1970, Kohn 1987). The selectivity of alginates for some divalent metal ions may be expressed quantitatively in terms of a 'selectivity coefficient' (Smidsrød and Haug 1968), that is, the affinity of a polyanion to a metal ion relative to the affinity to another metal ion.

The interaction of alginates with univalent cations (for example Na^+ , K^+ and Li^+) has been studied less extensively. However, after a series of experiments (Seale, Morris and Rees 1982), it has been suggested that three types of interaction may occur between univalent cations and alginates in solution:

- (i) weak chelation by mannuronate and isolated guluronate residues;
 - (ii) specific site-binding to adjacent guluronate residues;
 - (iii) co-operative 'egg-box' binding, especially of Na^+ ,
- between poly-L-guluronate sequences.

The binding of iron by alginate has potential significance in the food and pharmaceutical industries. Since alginate is not generally absorbed in the small intestine where most iron is absorbed into the body, there is a possibility that sufficient ingestion of alginate could lead to decreased bioavailability of iron. Attempts have been made to relate iron bioavailability to the presence of

dietary polysaccharides. For example, Harmuth-Hoene and Schlenz (1980) demonstrated a decrease in iron absorption in rats when sodium alginate, carrageenan or agar constituted 10% of the diet. Wölbling et al (1980) showed that an artificial diet containing 10% sodium alginate inhibited the absorption of iron in normal but not in iron-deficient rats. The *in vitro* binding of iron by sodium alginate has also been demonstrated (Berner and Hood 1983), although its effect on more complicated food systems is still unclear.

Toxic heavy metals such as lead, cadmium and strontium are health hazards in industrial areas where they are contained in airborne dust particles. On inhalation, they may be deposited in the respiratory tract and passed into the gastrointestinal tract. *In vitro*, there is evidence that alginates bind heavy metals, thus their possible uses in reducing absorption of these toxic chemicals in humans has been proposed. However, although *in vitro* and *in vivo* studies of binding of alginate to heavy metals are widespread, they are often contradictory.

For example, *in vitro* studies in isolated rat jejunum and duodenum demonstrated that the uptake and absorption of strontium is inhibited by alginates (Moore and Elder 1965, Patrick 1967). These results are confirmed by *in vivo* investigations (Carr and Nolan 1968). However, for lead, alginates have been shown to decrease absorption in newborn rats fed a milk diet, but to increase absorption over a three day period in adult rats fed a standard diet (Carr et al 1969).

According to Rose and Quarterman (1987), aqueous solutions of alginic acid bound lead and cadmium *in vitro*. *In vivo*, however, addition of alginic acid to the diet of rats was shown to increase lead retention, but had no effect on cadmium. In humans, no effect of alginates on the absorption of lead could be demonstrated (Harrison et al 1969).

As can be seen, the reported findings are inconclusive and, as yet, there is little information which suggests definite beneficial effects of dietary fibres such as alginates in reducing accumulation of

heavy metal in humans. However, as already mentioned, sodium alginate may be administered in a nuclear accident in order to bind radioactive strontium ions (section 1.2.2).

1.3.3 With Other Polysaccharides

Interactions between alginates and pectin were originally observed in the food industry. When a 2% solution of sodium alginate from *Laminaria hyperborea* was added to a fruit product such as apple purée, with a sugar content below 30%, it was found to form a heat-reversible gel, which led to the conclusion that an interaction had taken place between the pectin in the fruit and the added alginate (Toft 1982). Toft noted that the above gels demonstrated freeze-thaw stability and that the nature of the gel was influenced by pH, sugar concentration and hydrocolloid concentration.

Other workers reported that mixtures of alginates and pectins formed firm resilient gels at low pH, in the absence of calcium ions or high sugar concentrations (Steinnes 1975).

It has since been demonstrated that combinations of alginate and pectin produce gels under conditions at which neither polysaccharide forms gels independently (Oakenfull et al 1990). Stronger gels are produced from mixtures of pectins with high methoxyl content and alginates with high guluronate content in a ratio of pectin : alginate of approximately 1:1 (Thom et al 1982).

Thom and co-workers have proposed a theory for this interaction between alginates and pectins. The optimum interaction is found between homopolymeric sequences of L-guluronic acid residues in the alginate (Figure 1.8), and methyl esterified D-galacturonic acid residues in the pectin (Figure 1.9), at low pH in the absence of calcium ions. Molecular modelling has shown that these ribbons can pack together in parallel, two-fold, crystalline arrays with no cavities between the polymer chains. Each methyl group on the galacturonic acid chain can be opposed to the

Figure 1.8 L-guluronic acid residues (alginate)

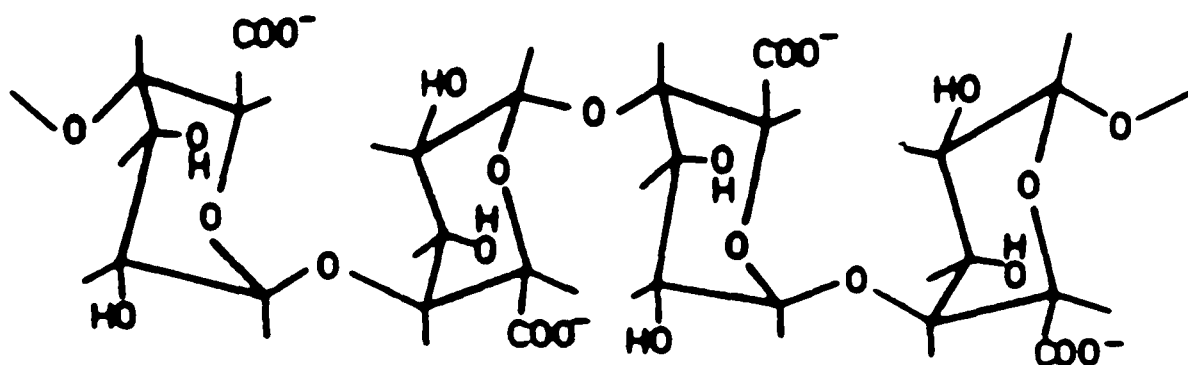
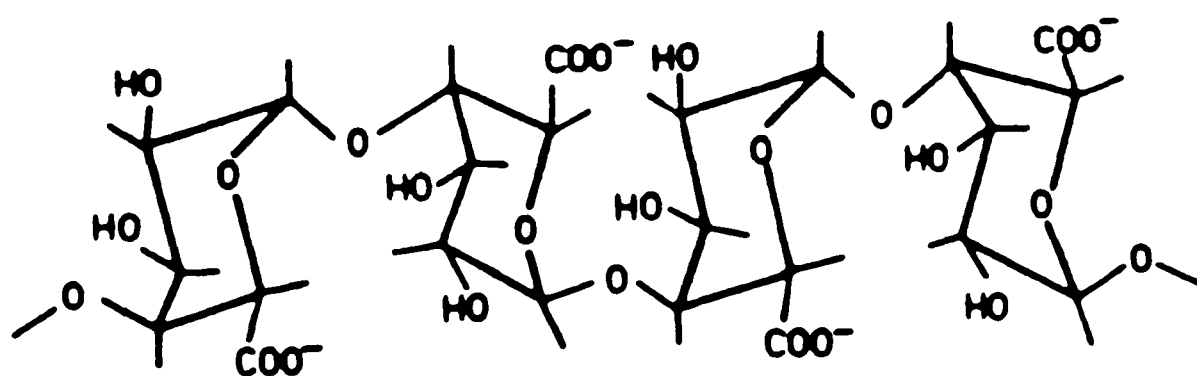


Figure 1.9 D-galacturonic acid residues (pectin)



hydrogen atoms attached to C(1) and C(2) of the guluronic acid residues, thereby minimising their interaction with water. Since the efficient packing of the chains requires minimal binding of water molecules for stability, the mixed structure is strongly favoured.

Both polyguluronate sequences and polygalacturonate sequences chelate calcium ions strongly to form 'egg-box' structures. Therefore, if calcium is present when the pectin/alginate mixture is being acidified, the ordered binding of alginate chains with other alginate chains, and pectin chains with other pectin chains, counteracts the formation of mixed junction zones. This explains why the addition of Ca^{2+} during the acidification process confers poor gelation properties. However, it has also been found that if pectin/alginate mixtures are first acidified to form a gel and then Ca^{2+} ions are dialysed in, even stronger gels are formed, probably by the formation of additional calcium-mediated junction zones which reinforce the original gel structure.

1.3.4 With Proteins

Interactions between proteins and polysaccharides occur frequently in biological systems, for example in such materials as the connective tissues of animals and in the membranes in certain eggs (Ledward 1979). Much of the reported work on protein-polysaccharide interactions has been carried out using ionic polysaccharides, as it has been demonstrated (Ganz 1974) that there is little or no interaction between proteins and non-ionic polysaccharides.

Imeson et al (1977) indicate that the major forces responsible for the interactions of myoglobin and bovine serum albumin with alginates, pectate and carboxymethylcellulose are electrostatic, involving the carboxylate groups of the polysaccharides and the positively-charged protein residues, for example amino and imidazole groups. Other workers, including Bernal et al (1987) and Gonçalves and co-workers (1986) have also demonstrated the

electrostatic nature of protein-polysaccharide interactions.

Stainsby (1980) suggested that it is possible that non-ionic interactions such as covalent bonds, hydrogen bonds or hydrophobic bonds may also have an important role in complex formation between proteins and polysaccharides. This is supported in a British Patent by Agfa (1964), who describes an interaction between propylene glycol alginate and gelatin under slightly alkaline conditions, which results in the formation of a gel with a melting point greater than 100°C. The predominant interactions in this gel would appear to have the thermal stability associated with covalent rather than ionic bonding.

Mohamed and Stainsby (1984) studied interaction and gel formation between propylene glycol alginate and various proteins including gelatin. They noted that the optimum conditions for gel formation were pHs in the range 9.3-9.6. Gels produced using untreated gelatin were found to be resistant to prolonged heat treatment. However, when the amino groups of the gelatin were converted to hydroxyl groups using nitrous acid, it was found that gels of much lower melting points were produced. Therefore, the interaction is proposed as being a covalent linkage between the polysaccharide and the protein, involving the amino groups on the gelatin. McKay et al (1985) further expand this theory. It was known that highly esterified alginates were required for the interaction (McDowell 1970), thus it is suggested that the most probable reaction is amide formation between the esterified carboxyl groups on the polysaccharides and the uncharged amino groups on the gelatin. The optimum pH range of 9.3-9.6 found by Mohamed and Stainsby (1984) supports this theory, since the number of uncharged amino groups present for interaction increases from about pH 9. However, beyond pH 9.6 the increase in uncharged amino groups and the resultant increase in reaction rate gives rise to the formation of grossly inhomogeneous gels because sufficient mixing of reagents is virtually impossible.

The addition of calcium ions to the polysaccharide/protein systems complicates the interactions, since the components may

then interact in more than one way. Both the polysaccharide and the protein could interact with Ca^{2+} ions. Alternatively, they could interact with themselves or with each other, with or without the involvement of Ca^{2+} ions (Hughes et al 1980). Guiseley (1980) suggests that, in solution, the anionic groups of ionic polysaccharides may interact not only with cationic molecules, but also with other anions via cation 'bridges' when divalent metal ions such as calcium are present.

In dilute solutions, complexing between proteins and polysaccharides usually occurs at pH values below the protein isoelectric point, that is, when the polyelectrolytes have opposite charges, so that complexation is mainly electrostatic in nature. However, addition of polyvalent metal ions such as Ca^{2+} may result in complex formation at pH values above the protein isoelectric point (Gluskey et al 1969, Hill and Zadow 1978). It is thought that the Ca^{2+} ions act as bridges, resulting in the formation of a triple complex of the form

PROTEIN - DIVALENT METAL ION - POLYSACCHARIDE.

Bernal et al (1987) added Ca^{2+} ions to the protein/polysaccharide systems described above, but did not suggest a specific molecular model for the interactions occurring in the mixed polysaccharide-protein-calcium ion gel systems.

Sherys et al (1989) investigated the formation of water insoluble triple complexes between bovine serum albumin and sodium alginate in the presence of bivalent metal cations, especially copper. They propose that the insoluble triple complexes

BOVINE SERUM ALBUMIN - CU(II) - ALGINATE

form due to bridging between the copper-binding sites of bovine serum albumin and alginate carboxyl groups via Cu^{2+} cations. However, the exact structure and mechanism of formation of triple complexes of this kind is not yet known.

1.4 PHARMACEUTICAL INTERACTIONS

1.4.1 Drugs and Excipients

In the food industry the numerous interactions of macromolecules in solution are viewed as an advantage, giving alginates and other hydrocolloids a variety of useful applications according to their individual properties (section 1.2). However, in the pharmaceutical industry, the interactions are seen traditionally as a disadvantage, since an active constituent of a preparation may interact with a macromolecular component in a manner that, for example, impairs drug release after administration, or inhibits preservative efficiency.

For example, it has been demonstrated that alginate binds to surfactants (Hayakawa et al 1983) which are often important stabilising components of pharmaceutical formulations. Interactions with alginate may reduce the efficacy of quaternary ammonium preservatives, and it is reported that cationic antiseptics, including acriflavine chloride and cetylpyridinium chloride become inactivated in 1% ammonium alginate gel as a result of complex formation (Keipert et al 1973).

Becirevic and Petricic (1980) studied the interaction of the antitussive noscapine chloride with various macromolecular agents including sodium alginate, using equilibrium dialysis, with a view to formulating oral liquid dosage forms. Their results indicate that noscapine chloride interacts with anionic species such as alginate. The authors view this interaction as a disadvantage, and it is suggested that 'in formulating a preparation that requires a combination of such materials, interacting macromolecular adjuvants should best be avoided'.

Habib et al (1991) reported an interaction between chloramphenicol and certain water-soluble macromolecules, such as polyethylene glycols, which affected the bioavailability of the drug and could therefore reduce its efficacy as an antimicrobial against *Streptococcus* bacteria.

Another drawback is that interactions may increase the resistance of certain bacteria to antibiotics. The exopolysaccharide of certain bacteria, for example, *Pseudomonas aeruginosa*, is a polyuronide similar in structure to alginates (Linker and Jones 1964). *Pseudomonas aeruginosa* is commonly found in patients with cystic fibrosis, a fatal inherited disease, and with other chronic lung diseases (Gill et al 1987).

In 1981, Slack and Nichols published findings that the diffusion of aminoglycoside antibiotics, for example, gentamicin and neomycin *in vitro* is retarded by sodium alginate and by the exopolysaccharide of a mucoid strain of *Pseudomonas aeruginosa*.

Tannenbaum and co-workers (1984), in apparent conflict with these findings, reported the inability of *Pseudomonas aeruginosa* to bind the antibiotics streptomycin, tobramycin, clindamycin and penicillin at physiological concentrations of saline, and therefore considered that the exopolysaccharide of *Pseudomonas aeruginosa* did not provide increased antibiotic resistance.

However, Nichols et al (1988) have now demonstrated that binding of the aminoglycoside antibiotic tobramycin does occur in the presence of alginate and exopolysaccharide from *Pseudomonas aeruginosa*, but that the level of binding which occurs in physiological buffer solutions is lower than that which could have been detected by the method of Tannenbaum et al (1984).

In vitro, this binding inhibits diffusion of the antibiotic to its target sites, probably by reducing the free concentration of the drug available for diffusion. These results have been supported by the findings of Gordon et al (1988), who reported that gentamicin and tobramycin 'bound avidly' to both commercial and *Pseudomonas* alginates.

1.4.2 Possibilities for Controlled Drug Release

Despite the apparent disadvantages of drugs interacting with macromolecular ingredients of a formulation, studies have been

undertaken which investigate the potential of using these interactions in order to control the delivery of various drugs.

For example, in 1961 and 1962, Graham and Thomas began studying the interactions of alginate, carrageenan and other hydrocolloids with alkaloids such as quinine, hyoscyamine, caffeine and theophylline. In a paper published in 1963, Graham et al considered that if highly insoluble complexes could be obtained from the interaction of various tranquilizers and hypotensive agents with hydrocolloids such as alginate, there was a possibility that controlled release forms of the drugs could be developed. Their interaction studies indicated that sodium alginate forms precipitates with the drugs promazine, chlorpromazine and reserpine, thus making the complexes 'potentially suitable as sustained release forms of these and possibly other drugs'.

More recently, Beldie et al (1989) have employed the interaction between neomycin and xanthan gum to form a delayed release complex, which was found to exhibit zero-order release kinetics when using artificial tear solution as the eluent. The authors suggest that it could be used to form ocular inserts for controlled release of drug.

Kawashima and co-workers (1989) prepared sustained release suppositories containing the antibiotic bacampicillin. In aqueous solution, the interaction of bacampicillin with alginic acid produced a slightly water-soluble complex, which was presumed to be linked by ionic bonding and was reportedly chemically stable. Simple physical mixing of sodium alginate with bacampicillin in a suppository base prolonged absorption of the drug in rabbits without a marked decrease in bioavailability compared to that after administration of bacampicillin alone. Furthermore, the absorption rate could be controlled by the amount of alginate added. The authors suggest that, after the suppository containing both substances dissolves, there may be simultaneous fast absorption of 'free' bacampicillin, together with formation of a bacampicillin/alginate ionic complex from which the drug is

released slowly over a prolonged period. Similar sustained- release suppositories containing morphine mixed with alginate were prepared (Kawashima et al 1990), which the authors suggest could be clinically useful, since prolonged absorptions were obtained by the addition of up to 50% alginate, without a decrease in bioavailability compared to that found in rabbits after oral administration.

Tucker et al (1988) studied the complexation of propranolol hydrochloride with carboxymethylcellulose. They suggest that with increasing concentration of propranolol, an increasing proportion of the carboxyl groups of the carboxymethylcellulose become occupied, until an insoluble complex is formed. Their binding data also indicated that there was more than one binding site, or that co-operative binding was occurring. That is, propranolol binds to the carboxyl groups on the carboxymethylcellulose until some fraction is occupied and precipitation occurs. Binding then continues to carboxyl groups on the precipitate. Tucker et al go on to suggest that such interactions may influence the bioavailability of a given product.

However, Hussain et al (1980b) incorporated methylcellulose into a nasal formulation of propranolol as a method of controlling release of the drug. They reported lower maximum blood levels of the drug (than when not incorporating methylcellulose), but that these were sustained over a longer period of time, such that there was an almost identical bioavailability.

This review has outlined the potential uses of macromolecular interactions in controlling drug delivery. The possibilities for other delivery systems employing macromolecules to achieve controlled release of drugs are considerable, and it is a field which obviously requires further investigation.

In the following work, we have studied the interaction of the anionic polysaccharide sodium alginate with propranolol hydrochloride, a model cationic drug, with a view to obtaining controlled release of drug both *in vitro* and *in vivo*.

1.5. AIMS AND OBJECTIVES

The overall aim of the work presented in this thesis was to achieve a greater understanding of the fundamental mechanisms controlling the interaction of an anionic polysaccharide polymer with an oppositely-charged drug molecule in an aqueous environment, and to investigate the potential of the ionic polysaccharide/drug complex formed as a controlled release drug delivery system.

Sodium alginate (from either *Laminaria hyperborea* or *Ascophyllum nodosum* species) was chosen as a model anionic polysaccharide and the following objectives were established in order to investigate its usefulness as part of a drug delivery system for controlled release:

1. To characterise of the alginates in terms of molecular weight, polydispersity and M:G ratio.
2. To investigate the interaction of the alginates with the model cationic drug propranolol hydrochloride and to study the effect of counter-ions such as sodium chloride on this interaction.
3. To quantify the interaction between the alginates and propranolol hydrochloride and to model the possible structure of any complex formed.
4. To prepare a convenient dosage form from the propranolol/alginate complex and to investigate the drug release properties of this formulation *in vitro* and in an *in vivo* animal model.

CHAPTER 2 CHARACTERISATION

2.1 INTRODUCTION - WHY CHARACTERISE?

A knowledge of molecular weights, molecular weight distributions, M:G ratio and polydispersity has been found to be of fundamental importance for many pharmaceutical and non-pharmaceutical applications of alginates. For example, in the food industry, the presence of low molecular weight 'tails' of alginate is detrimental to their use as gelling and thickening agents (Launay et al 1986). In the oil industry, polysaccharides are often added to fluids injected for mobility control in flooding operations, and the effectiveness of the polysaccharide in this context has been shown to be largely dependent on its molecular weight (Holzwarth 1985).

In the pharmaceutical field, polysaccharides are increasingly being used as potential drug delivery systems, for example, as carrier systems for drug targeting (Friend and Pangburn 1987), for sustained release (Melia 1991), and as bioadhesives for increasing the transit times of novel dosage forms in the small intestine (Anderson et al 1989). The performance and usefulness of these delivery systems depend greatly on the properties of the carrier system, which in turn depends in part upon the molecular characteristics of the polysaccharide used. In addition, any excipient which is to be used medically is required to be well characterised in order to avoid toxic reactions within the human body. Therefore, quite apart from the necessities of scientific investigation, when employing a polysaccharide in a drug delivery system, it is important that it is thoroughly characterised.

A systematic approach to the investigation of variables such as molecular weight, M:G ratio, polydispersity and polyelectrolyte behaviour for the manipulation and control of alginate interaction with specified drugs represents an excellent method for developing novel drug delivery systems.

The molecular weight and polydispersity of alginates are fundamental to their characterisation since they affect many other physical properties, for example, viscosity, osmotic pressure and gelation. These properties may influence the way in which an alginate delivery system releases drugs.

The M:G ratio of an alginate may also be important when studying possible complexes for drug delivery. It has already been demonstrated that G-blocks bind Ca^{2+} ions more strongly than M-blocks or MG-blocks (Grant et al 1973). Therefore, it is intended to study drug interactions with alginates possessing different M:G ratios, in order to investigate the possible usefulness of the M:G ratio as a tool for controlling drug delivery.

Finally, alginates are ionic, therefore the possibilities for electrostatic interactions with oppositely-charged drugs are countless. Electrostatic interactions may be easily altered by, for example, changes in solution pH or ionic strength. These are potentially useful drug release triggering mechanisms *in vivo*.

Two highly purified sodium alginates from Protan Laboratories, Norway were employed in this research project, which had been extracted from the brown seaweeds *Laminaria hyperborea* (batch number 902-282-904) and from *Ascophyllum nodosum* (batch number 911-211-04). The techniques used for characterisation of these alginates included:

- (i) gel permeation chromatography (with and without multi-angle laser light scattering);
- (ii) 'low speed' sedimentation equilibrium in the analytical ultracentrifuge;
- (iii) nuclear magnetic resonance spectroscopy.

In addition, the following information regarding the purity of the alginates was supplied by the manufacturer:

Product name:	<u>Laminaria hyperborea</u> <u>alginate</u>	<u>Ascophyllum nodosum</u> <u>alginate</u>
Batch number	902-282-04	911-211-04
Particles:	Filtered through 0.2 micron filter	Filtered through 0.2 micron filter
Protein content:	0.24%	0.12%
Metal ion content:		
Ca	23ppm	422ppm
Mg	7ppm	6ppm
Cu	2ppm	13ppm
Zn	20ppm	8ppm
Si	<1ppm	<0.5ppm
Sr	2ppm	45ppm
Fe	33ppm	55ppm
Mn	3ppm	<2ppm
Pb	10ppm	<10ppm
As	70ppb	82ppb
Hg	<10ppb	<30ppb

2.2 GEL PERMEATION CHROMATOGRAPHY / MULTI-ANGLE LASER LIGHT-SCATTERING (GPC/MALLS)

2.2.1 Introduction

Gel permeation chromatography (GPC) or gel filtration was first introduced in 1959 as a method for 'desalting and group separation' (Porath and Flodin 1959). Since then it has been used extensively in the purification and separation of many biological macromolecules, for example, enzymes, polysaccharides, nucleic acids and proteins.

In a typical GPC experiment, a column of known length and diameter is packed with a porous gel (the stationary phase) through which sample molecules pass suspended in an eluent, such as a buffer solution (the mobile phase). Movement through the gel bed depends on the flow rate of the mobile phase and the Brownian motion of the solute molecules which causes them to diffuse in and out of the stationary phase.

The separation of molecules in GPC depends on the different abilities of the sample molecules to enter pores in the gel. Very large molecules cannot enter the pores and thus move through the gel bed fastest. Smaller molecules which can enter the pores move more slowly throughout the column. Molecules are therefore eluted in order of decreasing molecular size.

Results from GPC experiments are usually presented as an elution profile, showing the variation of solute concentration in the eluent against the volume of eluent passed through the column. Other parameters obtained include V_0 , the void volume and V_t , the total volume. V_0 is the aqueous phase surrounding the gel beads and can be determined easily as it is the volume required for elution of a molecule so large as to be completely excluded from the gel pores. V_t is the total volume of the packed gel bed and therefore corresponds to the volume of eluent required to elute a molecule

small enough to be completely included on the column. Thus V_0 and V_t represent the exclusion limits of the column, since all molecules will be eluted between V_0 and V_t unless some other phenomenon, for example, adsorption is occurring.

V_0 and V_t can be determined experimentally using appropriate molecules. For the purpose of these experiments, blue dextran 2000 was used to determine V_0 , as it is both large and coloured, and is therefore also useful for checking correct packing of the gel bed since the colour allows a visual check that the sample is flowing in a straight band. Marshall (1970) points out that blue dextran 2000 should always be used fresh, as old solutions have been shown to bind to column materials. V_t was determined using sucrose, although other small molecules such as potassium chloride are suitable and are often employed.

2.2.2 Applications of Gel Permeation Chromatography.

The more important applications of GPC include: determination of molecular weights and molecular weight distributions; group separation and fractionation; separation of cells and particles.

GPC is an excellent technique for determining molecular weights and molecular weight distributions, provided that adequate average standards exist which enable the column to be calibrated for a given molecule, or that suitable absolute molecular weight detectors can be incorporated downstream of the column.. It has been particularly successful with proteins as it allows the molecular weight to be measured under a wide range of conditions of pH, ionic strength and temperature.

When determining molecular weight distributions, the elution profile may be recorded continuously or by collecting fractions and analysing them. Once a calibration curve has been determined for a

given macromolecule on a given column, it may be applied to a large number of runs, because the chromatographic behaviour of the column is extremely reproducible. However, the main problem is finding standards with which to calibrate the column. Typically, for polysaccharides, dextrans or similar compounds are used, but they are less suitable as standards for polysaccharides which differ greatly from dextrans in terms of their shape and conformation in solution.

Group separation involves separating molecules which differ greatly in size. Thus, applications include desalting of proteins and other large molecules prior to concentration, removal of free low molecular weight labels, for example, ^{125}I , from labelled molecules, and termination of reactions between macromolecules and low molecular weight reactants. Fractionation involves separating molecules of similar sizes and is therefore a useful technique for the purification of many biological macromolecules.

GPC has wide applications for separating cells and particles. For example, it has been used to separate erythrocytes from human blood (Kankura et al 1974), to remove monocytes from peripheral blood leucocytes (Alonso et al 1978), and also as a method for the preparation of platelets from blood plasma (Tangen et al 1971).

2.2.3 GPC/MALLS

Polysaccharide molecular weights are frequently determined by light scattering techniques. In traditional laser light scattering experiments, the sample is isolated in a cell, and there is one photomultiplier tube which is moved to different angles in order to measure the angular dependence of the scattered light.

A recent innovation has been to replace the isolated sample cell, containing a discrete quantity of solution, with a cell in the form of a glass block of narrow bore through which the solution flows at a constant rate. The angular dependence of the intensity of scattered light is measured by approximately 15 detectors situated around the cell. Thus, if the polysaccharide solution is first passed

through a GPC column (where it will have been fractionated according to molecular size) and from there passes into the sample tube described above, the molecular weight distribution of the polysaccharide can be determined. This technique is referred to as gel permeation chromatography/multi-angle laser light-scattering or GPC/MALLS.

2.2.4 Materials

Laminaria hyperborea alginate, BN 902-282-04 (Protan Laboratories, Norway);

Disodium hydrogen phosphate BN 71647162 (Fisons, Loughborough);

Potassium dihydrogen phosphate BN 33757060 (Fisons, Loughborough);

Sodium chloride BN 6794910K (BDH, Poole, Dorset);

Sephacryl S-400 (Pharmacia, Milton Keynes);

Phenol BN 108 (Fisons, Loughborough);

Sulphuric acid 95% (Fisons, Loughborough);

AnaLar deionised water BN 3137510L (BDH, Poole, Dorset).

All were used as received.

2.2.5 Methods

2.2.5.1 GPC

The principal alginate used for the work in this thesis, a highly purified sodium alginate produced from the *Laminaria hyperborea* species was used. The solvent used was a standard phosphate buffer of pH 6.5, containing Na_2HPO_4 and KH_2PO_4 made up to an ionic strength of 0.3 using NaCl in accordance with Green (1933). The gel used was Sephadex S-400 superfine.

The sodium alginate was made up to a volume of 5mL and applied to a column of Sephadex S-400 superfine (1.6cm internal

diameter x 85cm). The loading concentrations were approximately 0.4 mg mL^{-1} , and these were run in the phosphate-chloride buffer described above at a flow rate of approximately 10 mL h^{-1} . 2ml fractions were assayed for total sugar content using the phenol-sulphuric acid assay for total sugar content described by Dubois et al (1956). In summary, 1mL of 5%w/v phenol was added to sample volumes of 1mL and the mixture vortexed. 5mL of concentrated sulphuric acid was added vigorously to the liquid surface and it was vortexed again. The solvent was used as blank and after 30 minutes, absorbance at 485nm was recorded against the blank.

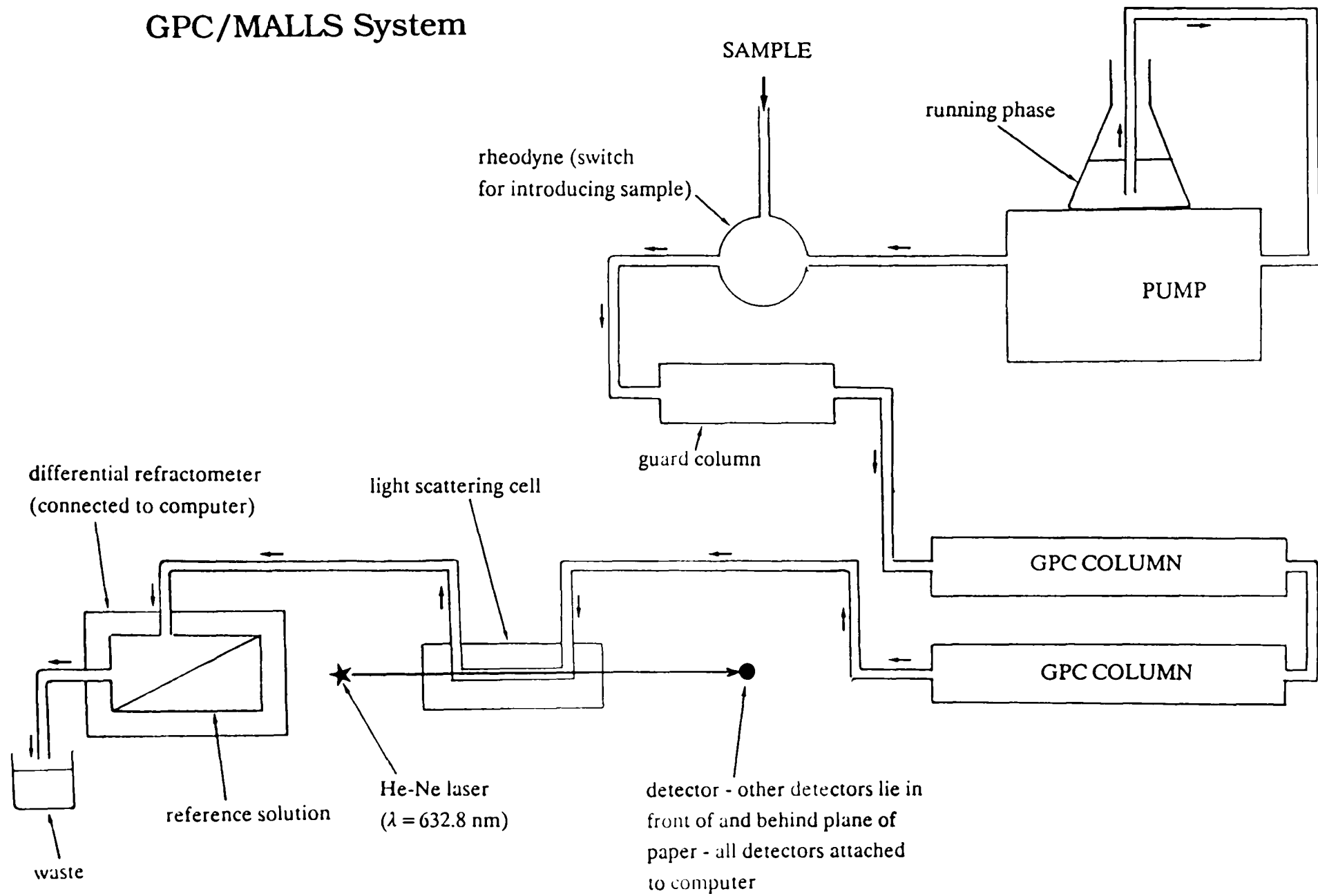
The void volume V_0 was determined using blue dextran 2000 and the total volume V_t using sucrose. Recoveries from the column were between 90 and 100%.

Each column run was performed in triplicate under the same conditions in order to check the reproducibility of the results. Standard deviations were found to be < 0.03 in all cases.

2.2.5.2 GPC/MALLS

A schematic diagram of the GPC/MALLS system used is shown in Figure 2.1. A Wyatt Technology Dawn F multi-angle laser light-scattering photometer was used to examine the molecular weight distribution of the purified alginate from *Laminaria hyperborea* mentioned above. Solutions of the sample ($0.5\text{-}5.0 \text{ mg mL}^{-1}$) were made up in the phosphate-chloride buffer described above, with an ionic strength of 0.1M or 0.3M. Buffer was then used as a mobile phase to wash the sample from the rheodyne, through the GPC column and into the light scattering cell. For data analysis it was necessary to know the concentration of the solution passing through the scattering cell at any given instant. This was achieved by measuring the refractive index of the solution using a differential refractometer placed after the light-scattering cell as shown in Figure 2.1. A computer was used to process and analyse the data from the GPC/MALLS system, in order to produce molecular weight distributions.

Figure 2.1 Schematic Diagram of GPC/MALLS System



2.2.6 Results and Discussion

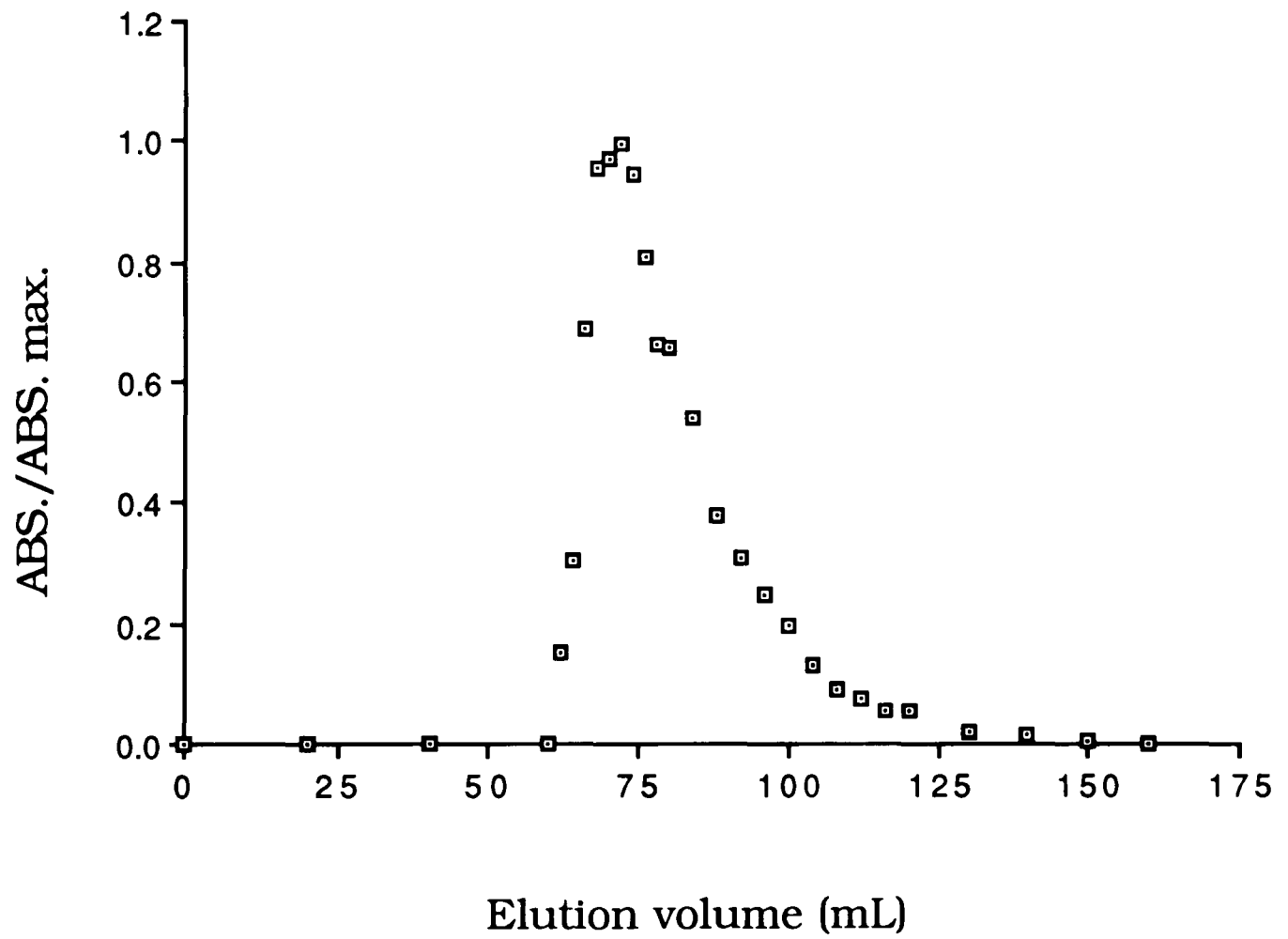
Figure 2.2 shows the elution profile of the highly purified *Laminaria hyperborea* alginate. The plot is normalised by plotting Absorbance/Maximum Absorbance versus Elution Volume in order to correct for small differences in the loading concentrations of the alginates. Values obtained for V_0 and V_t for the column used in these experiments were 60mL and 164mL respectively.

Phenol in the presence of sulphuric acid can be used for the quantitative determination of sugars, their methyl derivatives, oligosaccharides and polysaccharides. The phenol-sulphuric acid assay (Dubois et al 1956) was chosen for these experiments because it is simple, rapid, sensitive and gives reproducible results. It should be pointed out, however, that the sensitivity of the assay is such that even slight contamination of glassware can give rise to very high estimations of polysaccharide content. Therefore, as with all analytical work, a rigorous system of cleaning and drying equipment was essential. Despite this problem, the authors claim that 'under the proper conditions the method can be expected to be accurate to within $\pm 2\%$ '.

The elution profile is a function of an individual polysaccharide and therefore differs for different polysaccharides and indeed for different types of alginate. Valuable information concerning the polydispersity of compounds is gained from GPC: Figure 2.2 illustrates the polydisperse nature of the alginate, that is, the presence of non-interacting components of different molecular weight or composition.

For many GPC runs it is possible, using standards of known molecular weight, to convert the elution profile into a molecular weight distribution. Unfortunately, for alginates and many other polysaccharides, there are no adequate standards since they differ immensely in shape and conformation in solution compared to, for example, globular proteins.

Figure 2.2 Elution Profile of
L. hyperborea Alginate



Several possibilities have been suggested for overcoming this difficulty. For example, Ball et al (1988) proposed a technique for obtaining a molecular weight distribution for alginates using a combination of GPC followed by sedimentation equilibrium in the ultracentrifuge. However, this technique is time-consuming as it requires repeated GPC runs in order to obtain a sufficient concentration of sample for the subsequent sedimentation equilibrium experiments. A more rapid technique is that of GPC combined with multi-angle laser light-scattering (MALLS) as used in these experiments. In this case, the sample passes directly from an HPLC/GPC column into the light-scattering apparatus so that a molecular weight distribution may be obtained directly within hours as opposed to days.

Figure 2.3 is an example of a plot of the cumulative molecular weight distribution of the *L. hyperborea* alginate used above. Figure 2.4 shows the average molecular weights of the *L. hyperborea* alginate obtained by computer analysis of the GPC/MALLS data. It should be noted that the molecular weights obtained from this technique are virtually independent of loading concentration (compared to those obtained from sedimentation equilibrium in the following section 2.3). This is because the 'loading concentration' is a nominal value only, referring to the concentration of solution injected into the system. The solution becomes considerably diluted when it enters the rheodyne and meets the running phase of buffer.

Figure 2.3 Typical Cumulative Molecular Weight Distribution (*L. hyperborea* alginate) using GPC/MALLS

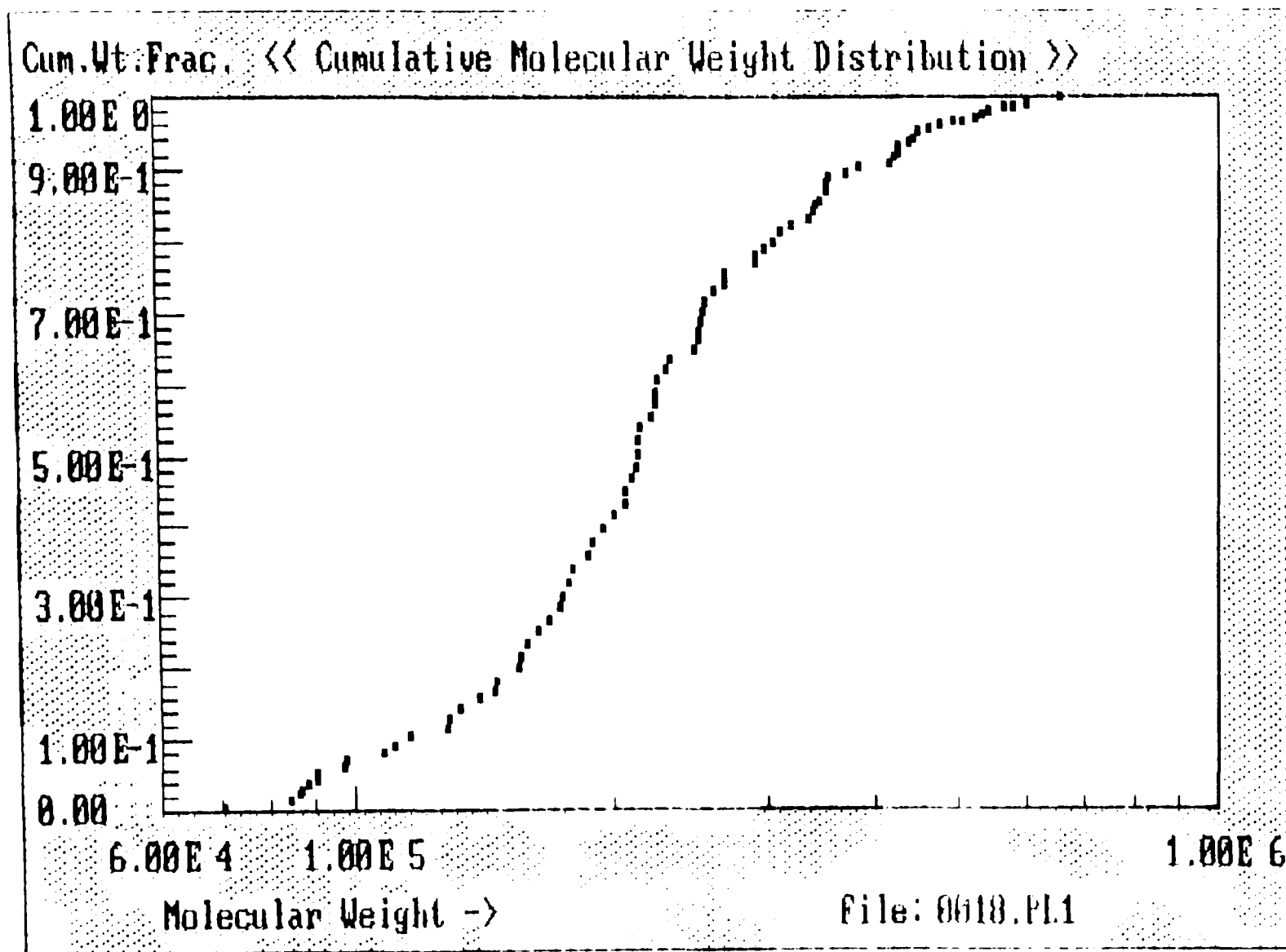
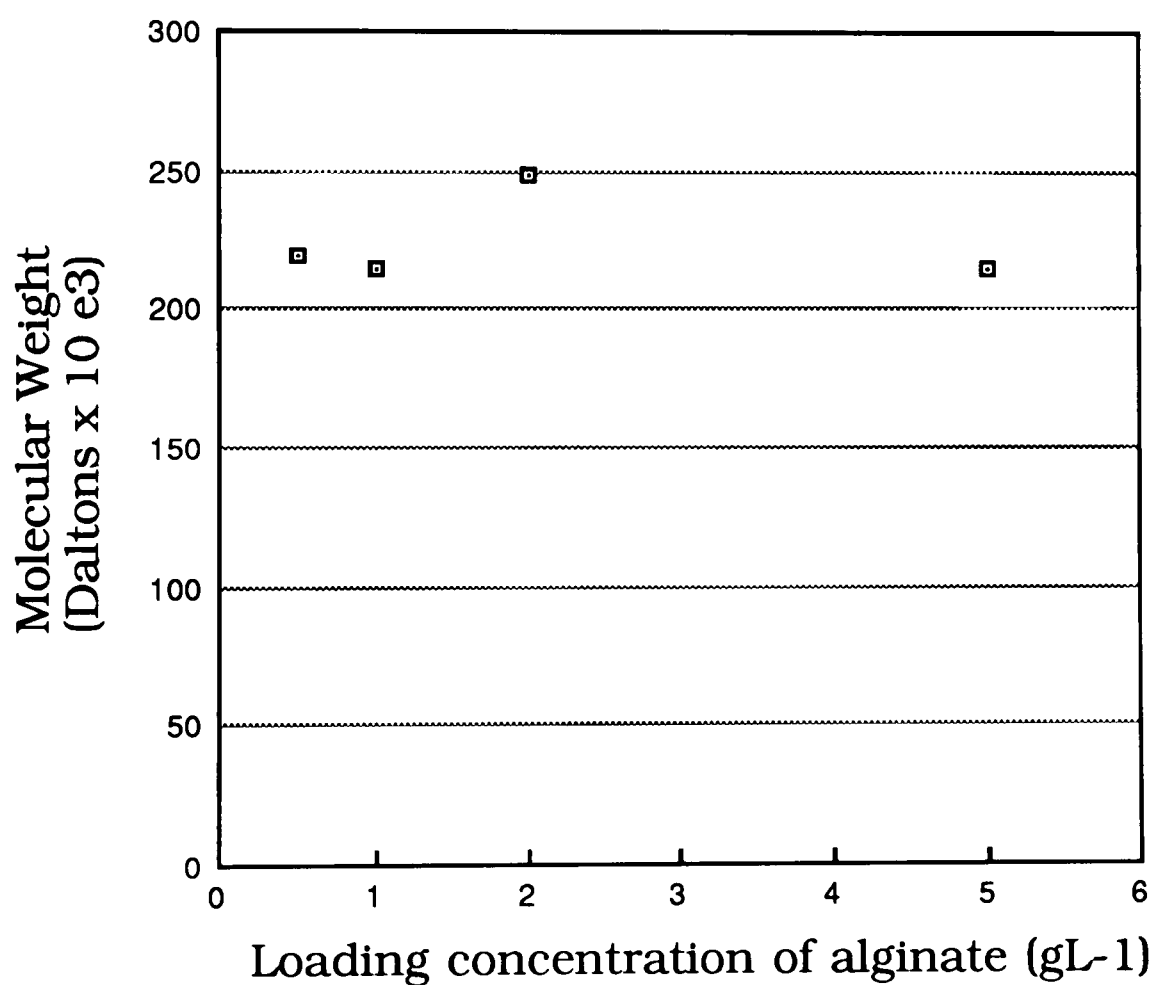


Figure 2.4 Molecular Weight of *Laminaria hyperborea* Alginate determined by GPC/MALLS (Buffer Ionic Strength = 0.3)



2.3 SEDIMENTATION EQUILIBRIUM IN THE ANALYTICAL ULTRACENTRIFUGE

2.3.1 Introduction

In order to characterise the alginates in terms of molecular weight, LOW SPEED SEDIMENTATION EQUILIBRIUM (LSSE) in the analytical ultracentrifuge was employed. This is an absolute technique, requiring no assumptions about shape or conformation in solution, and no calibration by standards of known molecular weight (Creeth and Harding 1982).

Inside the ultracentrifuge, a solution of the sample, together with a blank sample of solvent, is held in a two-sector cell inside a rotor. When the centrifuge is switched on, the sample molecules begin to sediment outwards from the centre of rotation. This tendency to sediment is balanced by diffusional forces counteracting the concentrating effect of the sedimentation process. In LSSE, the rotor is spun at relatively low speeds (2,000-15,000 r.p.m.) such that an equilibrium is set up between the sedimentational and diffusional forces described above. The position of the sample molecules within the cell is dependent on their molecular weight: under the centrifugal force, molecules of high molecular weight will sediment more rapidly than molecules of lower molecular weight, and will thus tend to accumulate nearer the base of the cell.

The distribution of the sample at equilibrium (after approximately 72 hours for the alginate samples used) is recorded using Rayleigh interference optics. From these distributions, information about the molecular weight of the sample can be obtained. The system works by shining a monochromatic beam of light simultaneously through the two sectors of the cell containing the sample solution and the pure solvent respectively. This produces 'interference fringes'. These fringes will be deviated from a straight line by any difference in the refractive index of the two solutions, for example that caused by the presence of solute molecules. Thus, it is

possible to observe a concentration distribution of solute across the cell, which is a direct reflection of the molecular weights of the individual species in the solution. Computer analysis of the fringes produced enables a 'weight-average' molecular weight to be calculated, that is, the mean molecular weight when components are counted by the 'weight' of each.

When using sedimentation equilibrium with alginates, the molecular weight observed has been found to be highly dependent on the concentration of the sample. To overcome this problem, the 'apparent' molecular weight is measured for a range of sample concentrations and then extrapolated to zero concentration to obtain the actual molecular weight. The relationship between apparent molecular weight and actual molecular weight may be expressed as follows (Harding and Johnson 1985):

$$1/M_{app} = 1/M + 2Bc \quad (1)$$

where: M_{app} = apparent molecular weight (in this case, that measured using sedimentation equilibrium);
 M = actual molecular weight;
 B = 2nd virial coefficient;
 c = concentration.

Therefore, a plot of $1/M_{app}$ vs. c would be expected to be linear, and extrapolating to zero should elicit the actual weight average molecular weight.

This approach has been taken in these experiments.

2.3.2 Materials

Laminaria hyperborea alginate, BN 902-282-04 (Protan Laboratories, Norway);

Ascophyllum nodosum alginate, BN 911-211-04 (Protan Laboratories, Norway);

Disodium hydrogen phosphate BN 71647162 (Fisons, Loughborough);

Potassium dihydrogen phosphate BN 33757060 (Fisons, Loughborough);

Sodium chloride BN 6794910K (BDH, Poole, Dorset);

AnaLar deionised water BN 3137510L (BDH, Poole, Dorset).

Beckman Model E analytical ultracentrifuge.

All were used as received.

2.3.3 Methods

The purified sodium alginates from *Laminaria hyperborea* and *Ascophyllum nodosum* species were used, dissolved in a standard phosphate-chloride buffer, pH 6.5, containing Na₂HPO₄ and KH₂PO₄ made up to an ionic strength of 0.3 using NaCl in accordance with Green (1933).

A Beckman Model E analytical ultracentrifuge was used, employing Rayleigh interference optics and an RTIC temperature measurement system. Molecular weight determinations were performed in 12mm or 30mm optical pathlength cells for a range of concentrations (0.2-2.4mg mL⁻¹) of sodium alginate. Prior to sedimentation equilibrium, each sample was made up in the solvent described above and dialysed against this solvent.

Initial sample concentrations were determined by dialysing a known volume of the original alginate-in-buffer solution against distilled de-ionised water to remove buffer salts, then drying to constant weight in a Gallenkamp vacuum oven at 70°C.

2.3.4 Results

Figure 2.5 and 2.6 are plots of apparent molecular weight versus concentration and 1/apparent molecular weight versus concentration respectively for the *L. hyperborea* alginate.

Similarly, Figure 2.7 and 2.8 are plots of apparent molecular weight versus concentration and 1/apparent molecular weight versus concentration respectively for the *A. nodosum* alginate.

Figure 2.5
Apparent Molecular Weight vs
Concentration for *L. hyperborea* alginate

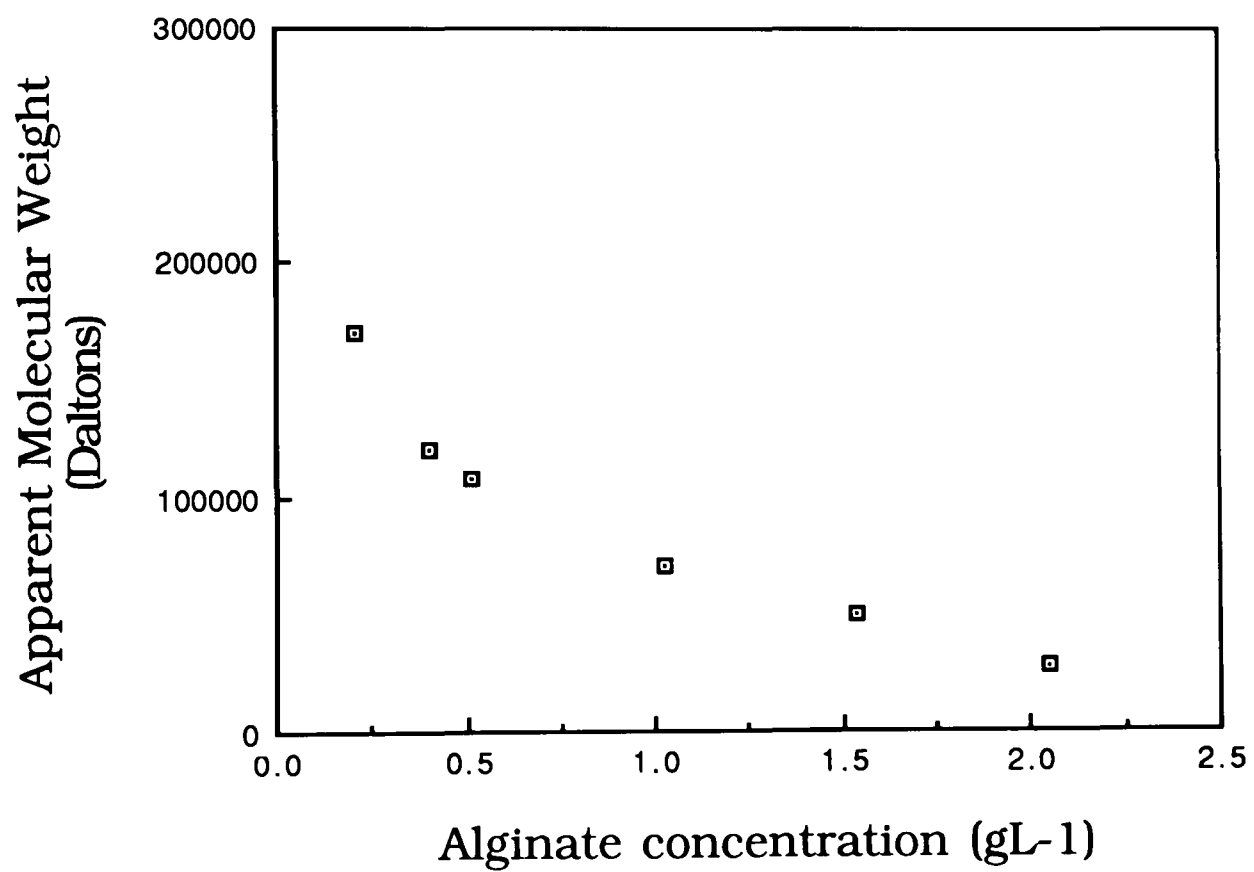


Figure 2.6
1/Mapp vs Concentration for
Laminaria hyperborea alginate

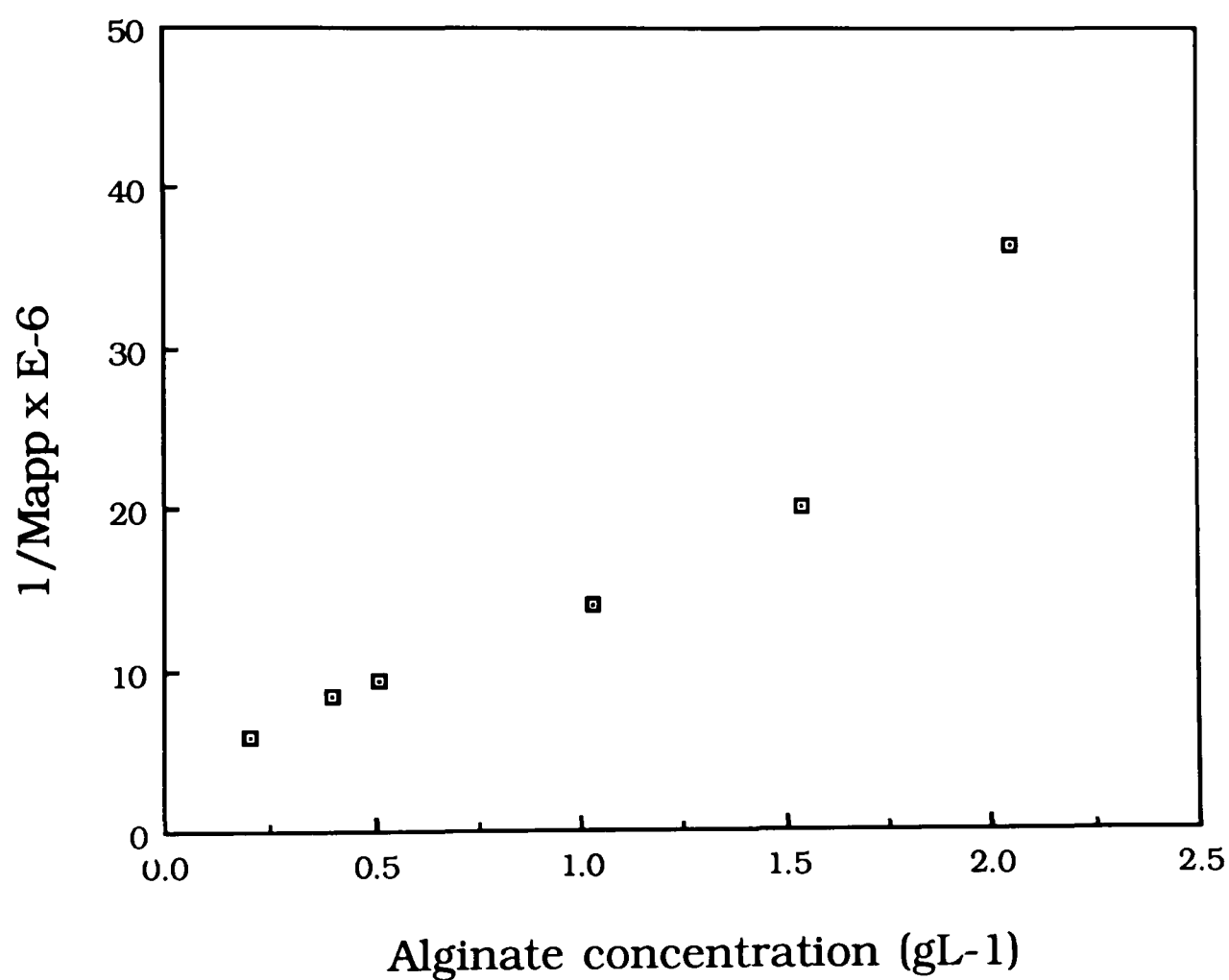


Figure 2.7
Apparent Molecular Weight vs.
Concentration for *A. nodosum* alginate

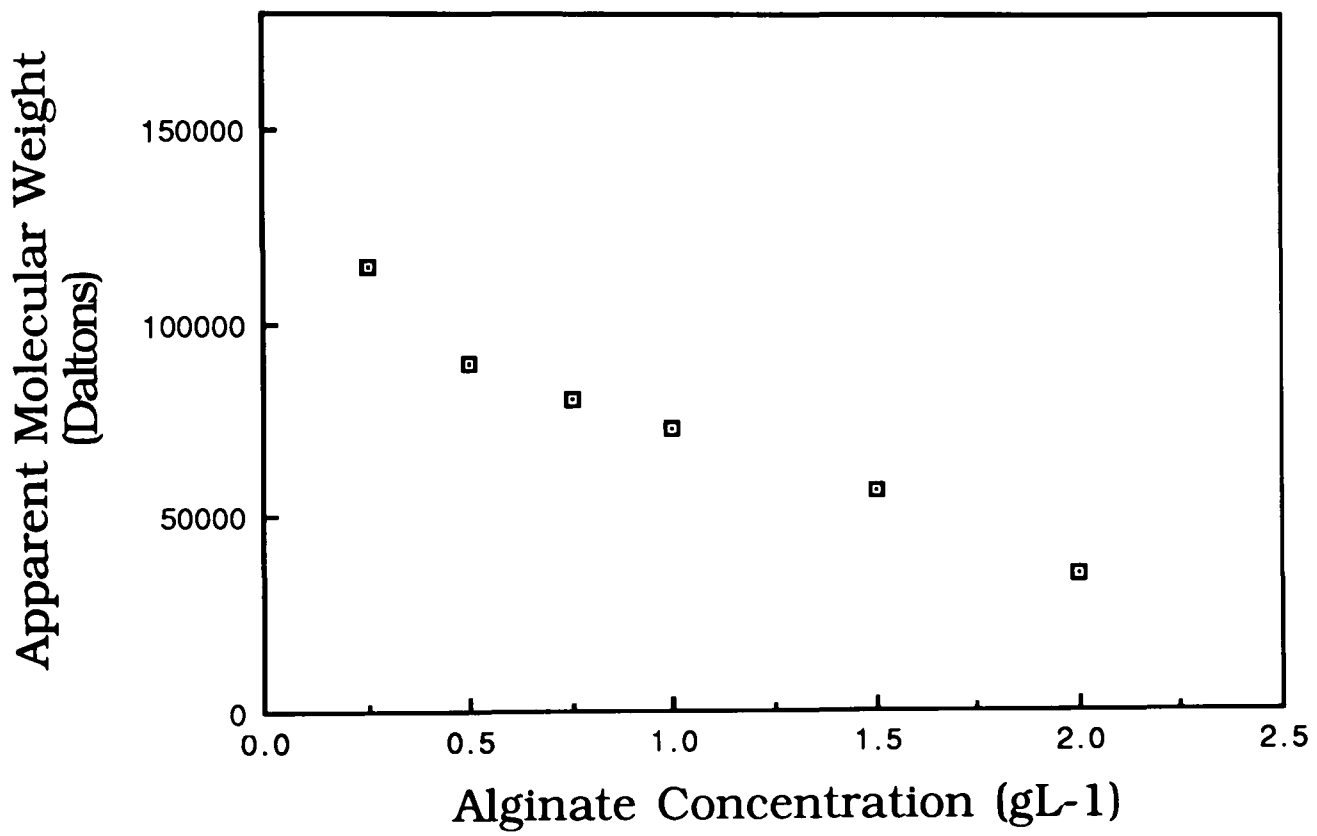
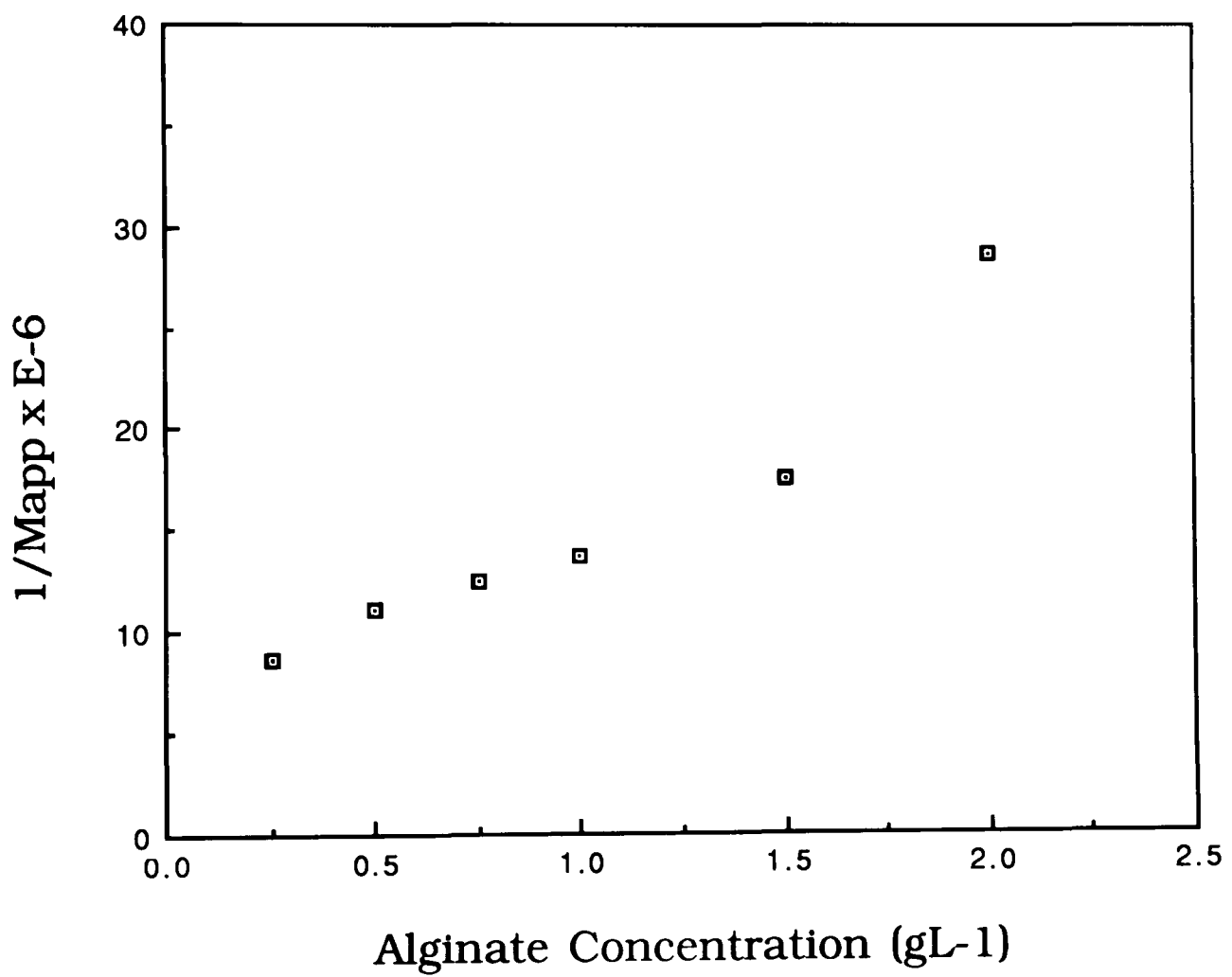


Figure 2.8
1/M_{app} vs. Concentration for
A. nodosum alginate



The plots of $1/M_{app}$ vs. concentration deviate from linearity above concentrations of around 1.0mg mL^{-1} . This is probably due to the fact that the effects of non-ideality for alginates are particularly severe (Harding et al 1992). Therefore, even at concentrations of around 1.5mg mL^{-1} , the systems do not behave in an ideal way, and do not fit equation (1), which is applicable to various other polysaccharide systems. However, below concentrations of 1.0mg mL^{-1} , the graphs are relatively linear and allow extrapolation of the plots to zero concentration in order to obtain weight-average molecular weights.

For *L. hyperborea*, the actual weight-average molecular weight obtained by extrapolation from the graph is 200,000-230,000 Daltons. For the *A. nodosum* alginate, the molecular weight obtained is 140,000-160,000 Daltons.

2.3.5 Discussion

Sedimentation equilibrium in the analytical ultracentrifuge as a means of determining polysaccharide molecular weights is not without its problems. A variety of factors contribute to the experimental difficulties:

2.3.5.1 Thermodynamic non-ideality

In general, polysaccharides are large molecules which may interact with solvent molecules or with each other, and therefore they have large thermodynamic exclusion volumes. In the case of alginate, the molecules are negatively-charged and thus also show polyelectrolyte behaviour.

One definition of an ideal solution is that it is a solution for which the law of ideal entropy of mixing holds. Entropy is a measure of the 'randomness' of a system, and this law states that, if there is no interaction between the molecules, the entropy of a mixture is

always greater than that of the pure components. The derivation of the law involves the assumption that all the solute molecules are of the same order of size as the solvent molecules, so that solvent and solute might be interchanged at random in a hypothetical lattice. However, this will not be the case for polysaccharides. Each macromolecule is many times larger than a solvent molecule so that a macromolecular solution better resembles one in which the solute particles are required to move together in clumps. Moreover, some polysaccharides self-associate and this effect is generally worsened at high solute concentrations. This can lead to gross over-estimation of molecular weight values.

For this reason, the distribution of solute molecules in a macromolecular solution can never be entirely random. The centre of each molecule is excluded from a volume determined by the volumes occupied by all the other molecules and these solutions are therefore termed 'non-ideal'. The problems of non-ideality may be minimised in the LSSE technique by employing low solute concentrations ($0.2\text{-}0.4\text{mg mL}^{-1}$) and long pathlength cells (Beckman 30mm).

Also, LSSE is highly dependent on the concentration of the original sample, which is itself difficult to determine accurately. For the purpose of these experiments, the original concentration was calculated by drying to constant weight in a vacuum oven but, at best, this gives a measurement of concentration of no better than 95% accuracy. Therefore, there is a degree of uncertainty as to the accuracy of the measurement, due to the problems of determining concentration efficiently. For this reason, the molecular weight measured is stated as being within a range of values (for example, for the *L. hyperborea* alginate this is 200,000 to 230,000 Daltons) as opposed to just a single value.

2.3.5.2 Polydispersity

Alginate consists of species of different molecular weights as

has been shown in the previous section on GPC (2.2). For this reason, molecular weights can only be expressed as averages. These may be:

(i) Weight average molecular weights (M_w), where the species in solution contribute according to their relative weights (as in LSSE);

(ii) Number average molecular weights (M_n), where the species in solution contribute according to their relative populations;

(iii) Z-average molecular weights (M_z), where the species in solution contribute according to the squares of their relative weights.

In the determinations carried out here using LSSE, the weight-average molecular weight was the principal parameter obtained, with the GPC and GPC/MALLS results giving a good indication of the polydispersity of the alginate.

2.3.5.3 Conformation.

The conformation of polysaccharides in solution is difficult to define in comparison to that of, for example, globular proteins. This may greatly affect the accuracy of those techniques for measuring molecular weight which require assumptions about shape and conformation in solution, for example, dynamic light scattering, gel permeation chromatography and viscometry. For this reason, LSSE and GPC/MALLS are both good choices for measuring the molecular weight of polysaccharides as no assumptions about shape or conformation in solution are necessary.

However, despite the problems described above, the molecular weight of 200,000 to 230,000 Daltons obtained by LSSE and by GPC/MALLS for the alginate from *L. hyperborea* compares favourably with estimations of its molecular weight using other techniques. Data from Paoletti (1989) on this alginate indicates the following results:

<u>Technique:</u>	<u>Molecular weight obtained:</u>
Wide angle light-scattering.	217,000
Low angle light-scattering.	214,000
Calibrated GPC	201,000.

2.4 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF ALGINATES

2.4.1 Introduction

Nuclear magnetic resonance spectroscopy (NMR), as its name implies, is concerned with the magnetic properties of certain atomic nuclei, notably the hydrogen atom or proton, and carbon ^{13}C . Studying an organic molecule by NMR spectroscopy enables the recording of differences in the magnetic properties of the various nuclei present, which gives information on their molecular environment and thus permits the deduction of the positions of the nuclei within the molecule. The theory of NMR is well documented (Kemp 1975) and it has become an extremely useful tool in the structural characterisation of organic molecules such as polysaccharides.

An important feature of alginate in terms of the way it interacts with certain counter-ions, for example, Ca^{2+} ions, is the M:G ratio. Using NMR, Grasdalen et al (1979, 1981 and 1983) have developed a method for characterising alginates which provides information not only about M:G ratio, but also allows the calculation of the average length of the G-blocks, the sites most important in binding with counter-ions (section 1.3.2). Each monomer unit, diad or triad within the alginate chain gives rise to a peak at a particular 'frequency' in the NMR spectrum, and the various peaks have been interpreted by Grasdalen et al. There are four possible diad frequencies: F_{GG} ; F_{GM} ; F_{MG} ; F_{MM} ; and eight possible triad frequencies: F_{GGG} ; F_{GGM} ; F_{MGG} ; F_{MGM} ; F_{MMM} ; F_{MMG} ; F_{GMM} ; and F_{GMG} . The peak areas can be used to calculate the monomeric composition (M:G ratio) of the alginate.

In the above series of papers, Grasdalen and co-workers demonstrated that alginates do not have a regular repeating unit.

Moreover, the sequential arrangement of the two monomers (mannuronic acid and guluronic acid) could not be determined by the monomer composition (monad frequencies) alone, but also required measurement of diad and triad frequencies. These values can be measured using NMR techniques, and from them the M:G ratio and the average length of the G-blocks can be calculated as outlined in section 2.4.1.2 and Appendix 2.

2.4.1.2 Equations

The relationship between the monad and diad frequencies is given by:

$$F_G = F_{GG} + F_{GM}$$

$$F_M = F_{MM} + F_{MG}$$

$$\text{and } F_{GG} + F_{MG} + F_{GM} + F_{MM} = 1$$

(It should be noted that for long chains where the contribution from the end groups can be neglected,

$$F_{GM} = F_{MG}).$$

For the triad frequencies (long chain alginates):

$$F_G = F_{GGG} + F_{MGG} + F_{GGM} + F_{MGM}$$

$$F_{GG} = F_{GGG} + F_{GGM}$$

$$F_{MG} = F_{GM} = F_{MGM} + F_{GGM}$$

$$F_{MM} = F_M - F_{MG,GM}$$

The average length of the G-blocks within the alginate molecule can be calculated from:

$$N_G = F_G / F_{MG}$$

2.4.2 Materials

Laminaria hyperborea alginate, BN 902-282-04 (Protan Laboratories, Norway);

Ascophyllum nodosum alginate, BN 911-211-04 (Protan Laboratories, Norway);

Hydrochloric acid (BDH, Poole, Dorset);

Sodium hydroxide BN 111 (Fisons, Loughborough);

AnaLar water BN 3137510L (BDH, Poole, Dorset);

Leybold-Heraeus 'Lyovac GT2' freeze-drier;

Bruker WM-500 NMR spectrometer.

2.4.3 Methods

The following method for alginates gave a degree of polymerisation of approximately 30-50 which ensured that the viscosity of the sample was not too high. The pH was reduced in stages, which prevented precipitating out of the alginate, which occurred if the pH was reduced in one large step.

An alginate (100mg) from either *Laminaria hyperborea* or *Ascophyllum nodosum* species was dissolved in approximately 25mL of deionised water. The pH was adjusted slowly to 5.4 with 0.1M HCl. This was then heated in a water bath at 100°C for 1 hour. After cooling to room temperature the pH was adjusted to 4.6 with 0.1M HCl. The solution was reheated in a water bath at 100°C for 1 hour, then cooled to room temperature and the pH adjusted to 6.8 with 0.1M NaOH.

The alginate solution was then freeze-dried using a Leybold-Heraeus 'Lyovac GT2' drier. (The sample was placed in a round-bottomed flask, swirled in 'dry-ice'-in-acetone until frozen, then transferred to the freeze-drier for approximately 12 hours until dry). It was then re-dissolved in D₂O ('heavy water') at a concentration of 10mg in 4mL D₂O. (This prevents the peak for

water in the NMR spectra obscuring other sample peaks and therefore provides better resolution). The sample was finally freeze-dried again as described above.

The proton spectra were recorded at 92°C using a Bruker WM-500 spectrometer.

2.4.4 Results and Discussion

The NMR spectra of the *Laminaria hyperborea* alginate and the *Ascophyllum nodosum* alginate are shown in figures 2.9 and 2.10 respectively.

The monomer composition, diad and G-centred triad frequencies were determined from the ¹H-spectra as described by Grasdalen et al (1983). The area under the peaks on the NMR spectra were determined using computer software at the University of Trondheim, Norway. Calculation of the M:G ratio and the average length of the G blocks is outlined in Appendix 2.

Tables 2.1 and 2.2 illustrate the composition and sequence parameters for the *L. hyperborea* alginate and the *A. nodosum* alginate respectively. These results indicate that the alginate from *L. hyperborea* has an M:G ratio of 35:65 and an average length of G-blocks of 6.2 residues, while the alginate from *A. nodosum* has an M:G ratio of 61:39 and an average length of G-blocks of 2.5 residues.

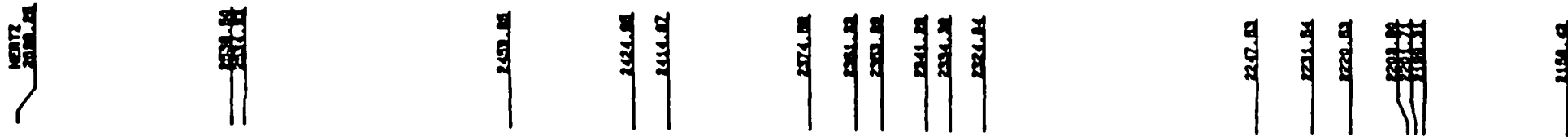


Figure 2.9
NMR Spectrum of *L. hyperborea* alginate



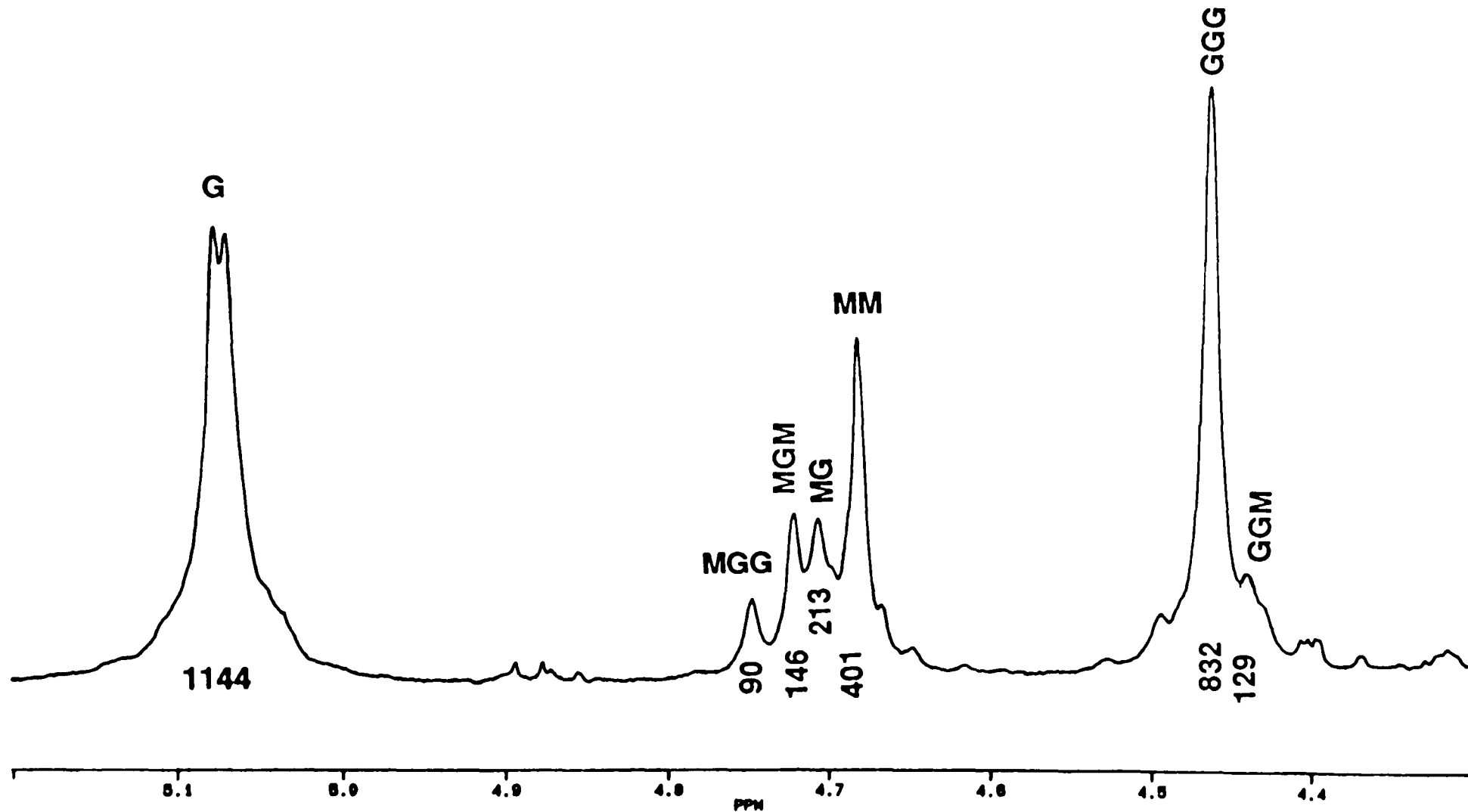
88BEX.005
DATE 11-9-89

RF 500.137
SY 83.0
O1 9200.000
SI 32768
TD 32768
SM 5000.000
HZ/PT .305

PW 7.0
PD 1.000
AQ 3.277
RG 40
NS 128
TE 297

FW 6300
O2 20000.000
DP 60L P0

LB .300
GB 0.0
CX 95.00
CY 0.0
F1 5.201P
F2 4.300P
HZ/CM 12.870
PPM/CM .028
SR 8960.01



PPM
 5.23104
 5.22581
 5.20132
 5.19451

 5.04176

 4.87683
 4.87552
 4.85407
 4.84892
 4.83199
 4.81196

 4.76433

 4.72509

 4.69833
 4.68101

 4.65465
 4.64085

 4.61209
 4.59492
 4.57207

 4.52333
 4.51986
 4.51547
 4.51108

 4.45966
 4.43715
 4.41483

 4.37400
 4.34823

Figure 2.10
NMR Spectrum of *A. nodosum* alginate



BAARDH.001
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SF 500.137
SY 83.0
O1 8290.000
SI 32768
TD 32768
SW 4310.345
HZ/PT .263

PW 9.0
RD 0.0
AD 3.801
RG 16
NS 224
TE 297

FW 5400
O2 20000.000
DP 60L P0

LB .130
GB .150
CX 35.00
CY 0.0
F1 5.245P
F2 4.339P
HZ/CM 12.944
PPM/CM .026
SR 6958.80

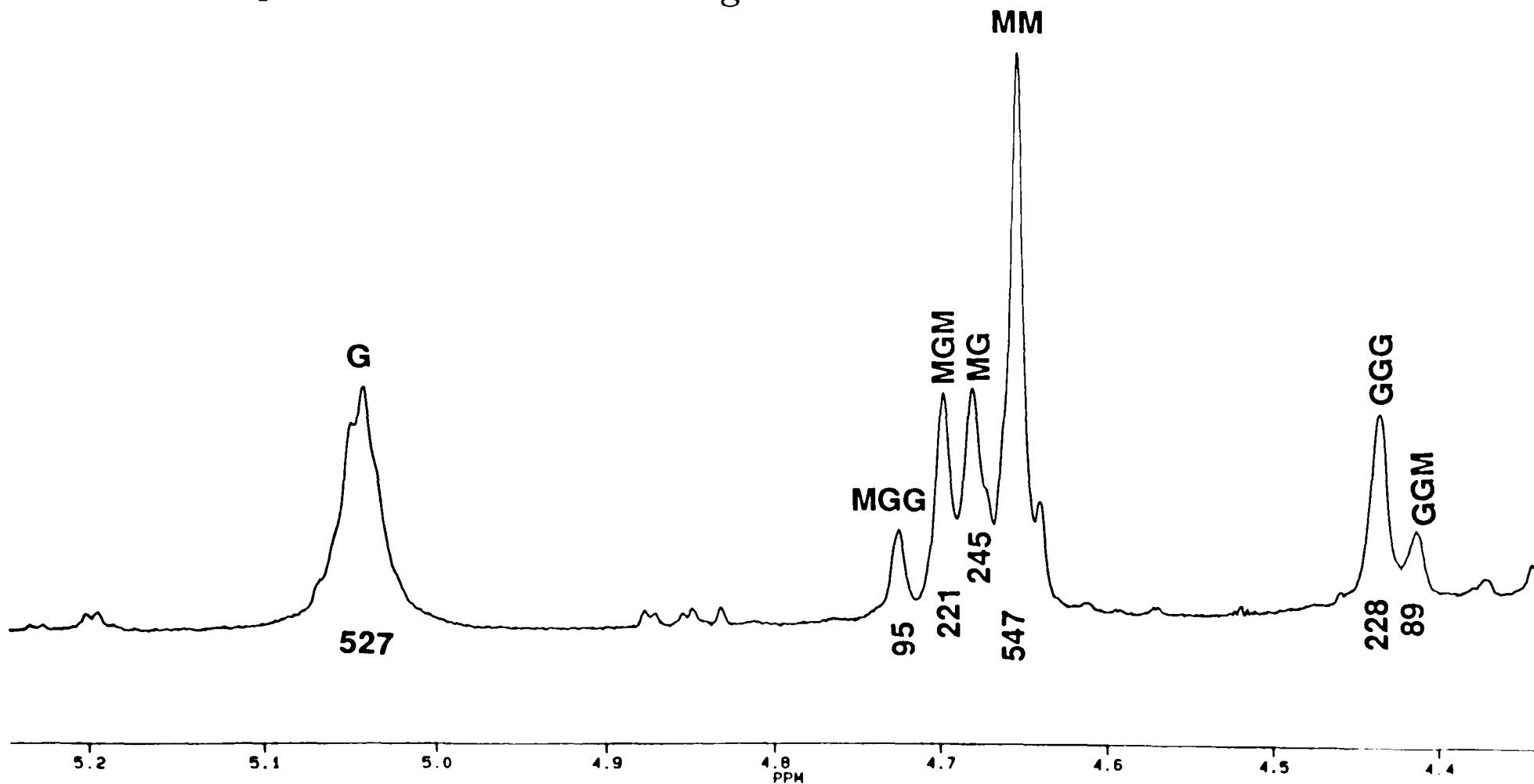


Table 2.1 Composition and Sequence Parameters
for *L. hyperborea* alginate
BN 902-282-04

FG	0.647
FM	0.353
FGG	0.543
FMM	0.249
FGM, MG	0.104
FGGG	0.470
FGGM	0.073
FMGM	0.031
NG (average length of G-blocks)	6.2
M:G ratio	35 : 65

Table 2.2 Composition and Sequence Parameters
for *A. nodosum* alginate
BN 911-221-04

F_G	0.389
F_M	0.611
F_{GG}	0.234
F_{MM}	0.456
$F_{GM, MG}$	0.155
F_{GGG}	0.168
F_{GGM}	0.066
F_{MGM}	0.089
N_G (average length of G-blocks)	2.5
M:G ratio	61 : 39

2.5 CONCLUSIONS

2.5.1. *Laminaria hyperborea* alginate

(Protan Laboratories, batch number 902-282-04)

This alginate has been characterised as follows:

- (i) Weight-average molecular weight: 200,000-230,000 Daltons (from LSSE, light-scattering and calibrated GPC measurements);
- (ii) Molecular weight distribution: refer to Figures 2.2 and 2.3;
- (iii) M : G ratio = 35 : 65;
- (iv) Average length of G blocks: 6.2 residues.

2.5.2 *Ascophyllum nodosum* alginate

(Protan Laboratories, batch number 911-221-04)

This alginate has been characterised as follows:

- (i) Weight-average molecular weight: 140,000-160,000 Daltons (from LSSE measurements);
- (ii) M : G ratio = 61 : 39;
- (iii) Average length of G-blocks: 2.5 residues.

CHAPTER 3

VISCOMETRY AND NEPHELOMETRY

3.1 VISCOMETRY

3.1.1 Introduction

The formation of molecular complexes by soluble polymers in solution is usually associated with a modification of rheological behaviour (Keipert et al 1973). The addition of multivalent cations, for example calcium ions, to solutions of alginates increases the viscosity of those solutions as a result of interchain crosslinking (Smidsrød and Haug 1972b; Penman and Sanderson 1972). At the outset of these studies, it was thought that the addition of a positively-charged drug to an alginate solution could have a similar effect, thereby producing a drug trapped within an alginate gel, which could form the basis of a drug delivery system. Therefore, the following experiments were designed to measure the viscosity of alginate solutions, and to investigate the effect of adding a model positively-charged drug, propranolol hydrochloride, to those solutions.

Viscosity is the property of a fluid which mainly characterises its 'flow' behaviour. The concept of viscosity involves the idea of internal 'friction' between molecules of the fluid, such that, whenever any part of the fluid is caused to move, neighbouring parts tend to be carried along also. This creates a resistance to 'flow', which forms the basis of the quantitative measurement of viscosity. The presence of macromolecules in a solution generally has the effect of increasing the resistance to flow of that solution compared with the pure solvent, which can be measured as an increase in the viscosity of the solution using a suitable viscometer.

The most common viscometers used are capillary viscometers, since they are comparatively simple, inexpensive, and require only a small amount of test liquid. Since the viscosity of a liquid usually decreases with an increase in temperature, often by an appreciable amount (Dinsdale 1962), temperature control is essential, and is again relatively easy with a capillary viscometer.

The viscometer used for these experiments was a U-tube capillary viscometer as described in the British Pharmacopoeia 1988.

3.1.2 Materials and Equipment

U-tube viscometer size B, type PSL BS/U no. 1353, made of clear borosilicate glass complying with specifications to

International Standards ISO 3104-1976 and 3105-1976;

Viscometer bath (Townson and Mercer Ltd., Croydon);

Laminaria hyperborea alginate (highly purified), BN 902-282-04

(Protan Laboratories, Norway);

Laminaria hyperborea alginate (non-purified), BN 902-282 (Protan Laboratories, Norway);

Propranolol hydrochloride BN 8905237 (CP Pharmaceuticals, Loughborough);

Sodium chloride, BN 6794910K (BDH, Poole, Dorset);

AnaLar deionised water BN 3137510L (BDH, Poole, Dorset).

All reagents were used as received.

3.1.3 Methods

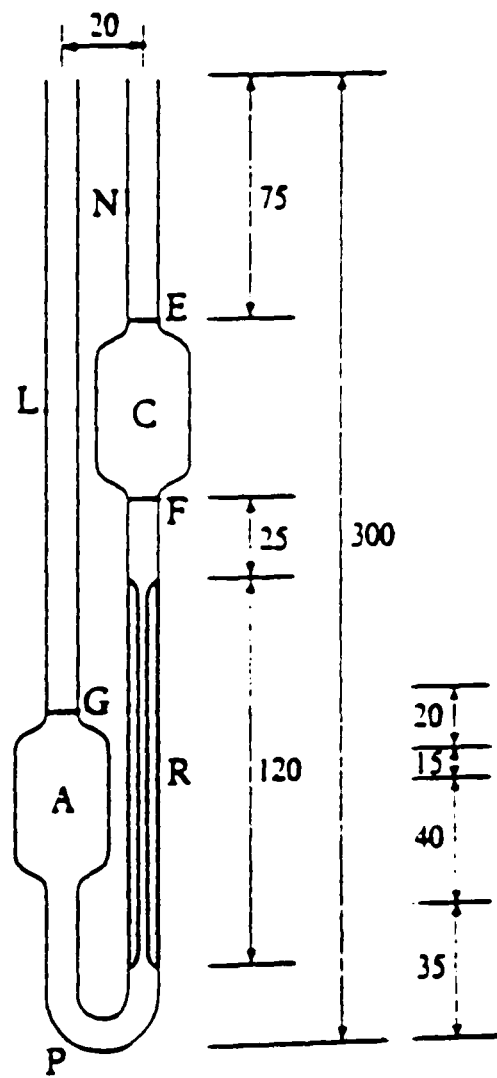
The size of the viscometer to be used was determined by measuring the run times of sodium alginate solutions at concentrations of 0.01gL^{-1} , 0.1gL^{-1} and 1.0gL^{-1} , in viscometer sizes A, B, C and D. A size B viscometer was chosen for these experiments as it yielded run times of greater than 60 seconds for the lowest concentration of alginate being measured, but less than 15 minutes for the highest concentration (B.P. 1988).

Viscosity determinations were carried out as described in the British Pharmacopoeia 1988, A100, Method 1. The apparatus used is illustrated in Fig 3.1. To summarise, the viscometer was filled with the liquid being examined through tube L to slightly above the mark G. The tube was placed vertically in a viscometer bath at 25°C ($\pm 0.1^\circ\text{C}$), and left to equilibrate at this temperature for 15 minutes. The volume of liquid was adjusted so that the bottom of the meniscus settled at the mark G. The liquid was then sucked to a point approximately 5mm above the mark E. On releasing the suction, the time taken for the bottom of the meniscus to fall from the top edge of mark E to the top edge of mark F was measured. The mean value of five readings was taken.

The viscosity of solutions of macromolecules is conveniently expressed by the ratio of the viscosity of the solution, η , to the viscosity of the pure solvent, η_0 . This ratio is referred to as the 'relative viscosity', so that: $\eta_{\text{rel}} = \eta / \eta_0$. The mean run-time ($n = 10$) of AnaLar deionised water (in the size B U-tube viscometer used in these experiments) was determined as 68.2 seconds by the method described above. All further run times of the alginate/ propranolol mixtures were converted into relative viscosities by comparison with this run-time value for water.

Initially, solutions of both a non-purified and a highly-purified *Laminaria hyperborea* alginate (0.01gL^{-1} to 1.0gL^{-1}) were examined. (The 'non-purified' was a standard industrial grade alginate, and the 'highly-purified' was the same alginate which had undergone a rigorous purification process to remove contaminants such as polyphenols (section 1.1.5)). The effect of adding propranolol hydrochloride (0.00025M - 0.1M) on the viscosity of 0.5gL^{-1} and 1.0gL^{-1} alginate solutions was then investigated. As a control, sodium chloride was added to the alginate solutions in the same proportions as propranolol hydrochloride.

Figure 3.1 U-tube Viscometer
(BP 1988)



U-Tube Viscometer
Dimensions in mm

3.1.4 Results

Figure 3.2 shows the relative viscosities for solutions of *Laminaria hyperborea* alginates (purified and non-purified) in AnaLar deionised water.

Figures 3.3 and 3.4 show the effect of adding propranolol hydrochloride to the alginate solutions, compared with the effect of adding sodium chloride solution (control), for the non-purified and purified alginate respectively.

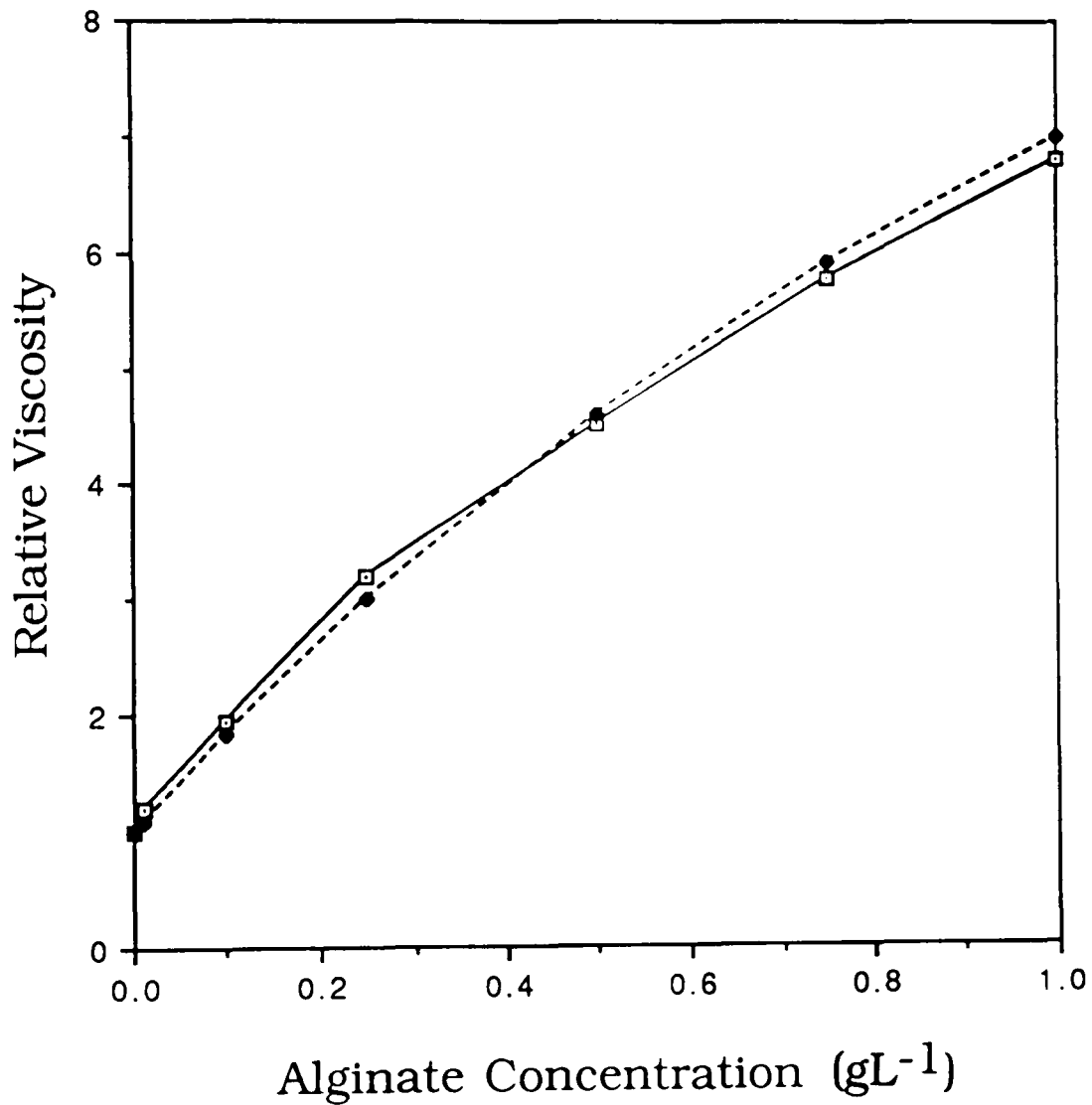
3.1.5 Discussion

From Figure 3.2, it can be seen that increasing the concentration of alginate in the solution increased its viscosity as is expected, due to the presence of increasing numbers of the highly-extended alginate molecules restricting the 'flow' of the solution.

For both types of alginate, the addition of propranolol or sodium chloride caused a decrease in the viscosity of the alginate solutions. In general terms, this may be explained in terms of the shape of the alginate molecules in solution, since, to a large extent, this determines the flow properties, and therefore the viscosity, of the solution. The shape of the flexible charged alginate molecules will vary depending on the degree of ionisation. Therefore, in an aqueous solution, the alginate molecules will be expanded due to mutual charge repulsion between the alginate molecules and the water molecules, and the viscosity increases. On addition of small counter-ions such as sodium ions, the effective charge is shielded and the alginate molecules contract causing the viscosity to decrease (Smidsrød 1979).

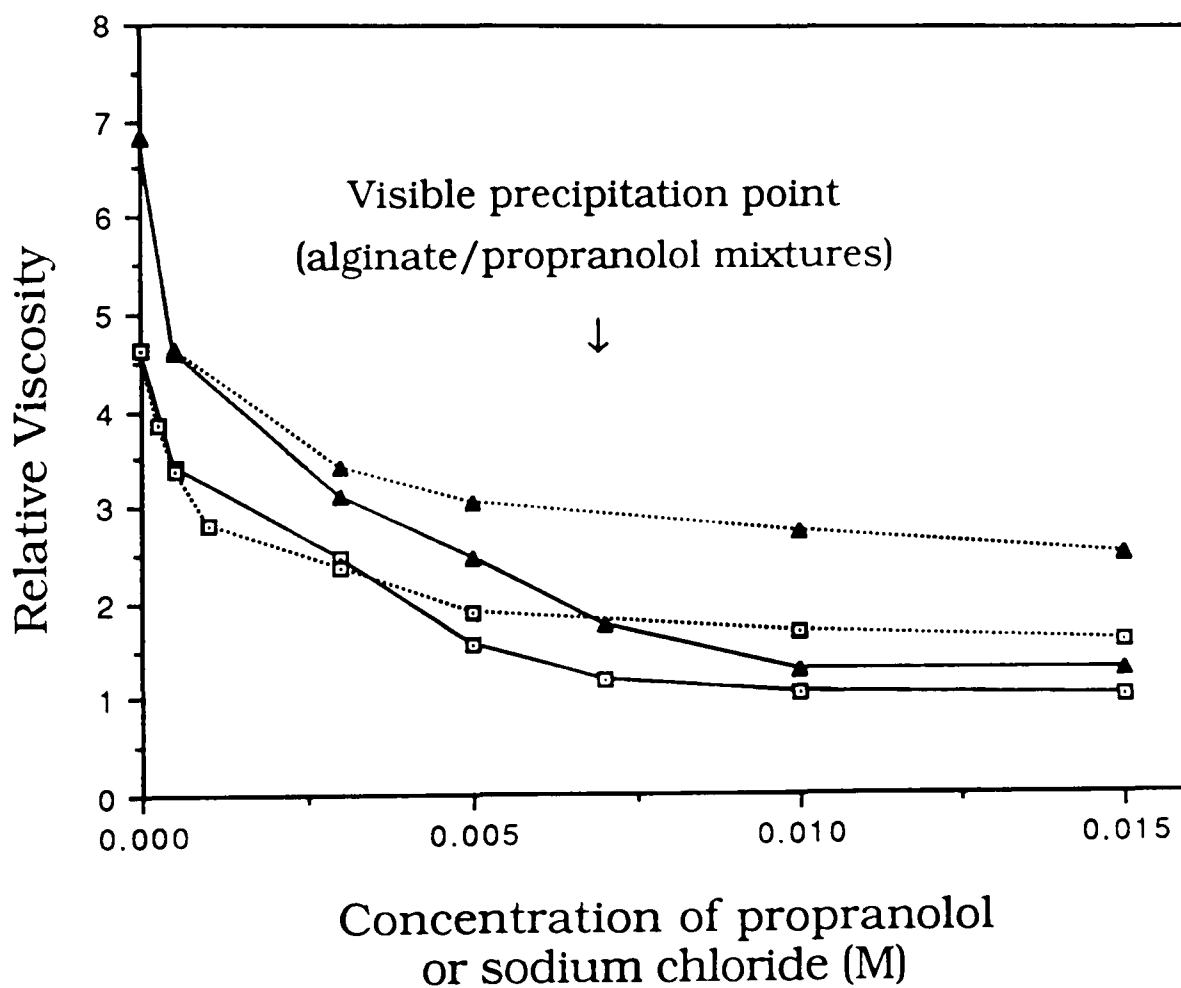
However, the viscosity decrease on addition of propranolol was greater than that observed on addition of sodium ions (control) at the same concentration (Figures 3.3 and 3.4). It was observed that, above a certain concentration of propranolol (approximately

Figure 3.2 Viscosity of Non-Purified and Purified *L. hyperborea* Alginate Solutions



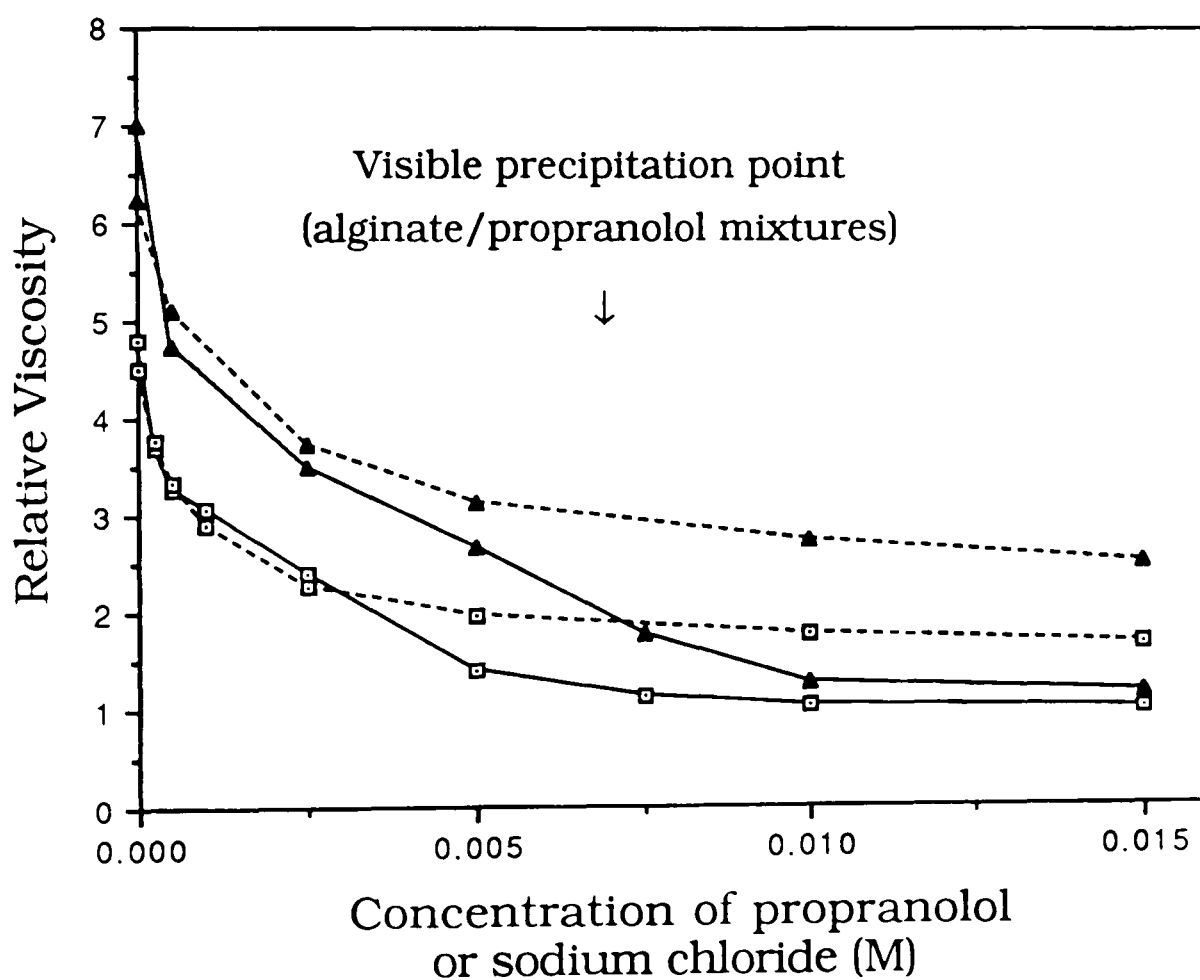
—□— Non-purified
- - -●- - Purified

Figure 3.3
 Viscometry of Alginate/Propranolol and
 Alginate/Sodium Chloride Mixtures:
 Non-Purified *L.hyperborea* Alginate



- Alg. 0.5g/L-1 + propranolol
- ▲— Alg. 1g/L-1 + propranolol
-□..... Alg. 0.5g/L-1 + NaCl
-▲..... Alg. 1.0g/L-1 + NaCl

Figure 3.4
 Viscometry of Alginate/Propranolol and
 Alginate/Sodium Chloride Mixtures:
 Purified *L. hyperborea* Alginate



- Alg. 0.5g/L + Propranolol
- ▲— Alg. 1.0g/L + Propranolol
- - - □ - - - Alg. 0.5g/L + NaCl
- - - ▲ - - - Alg. 1.0g/L + NaCl

0.007M when mixed with the 1gL^{-1} alginate solutions), a white-coloured precipitate was formed. This phenomenon was not observed in the alginate/sodium chloride mixtures, therefore it was concluded that an interaction was occurring between the alginate and propranolol hydrochloride which was causing a precipitate to be formed. In this case, two effects contribute to the decrease in viscosity observed. There is charge shielding (as described above for addition of sodium ions above) but, in addition, there is a decrease in solubility, resulting in the formation of a precipitate and a further decrease in viscosity as the polymer is removed from solution. Thus, the alginate/propranolol mixture behaves as a relatively non-viscous liquid (with a relative viscosity almost equal to that of the pure solvent) containing particles of precipitate.

It was decided to examine various mixtures of alginate with propranolol in order to study the formation of the precipitate observed in the previous experiments, and to quantify the point of precipitation for different types of alginate. The technique chosen to perform this was nephelometry.

3.2 NEPHELOMETRY

3.2.1 Introduction

Nephelometry, a simple but sensitive technique for measuring the turbidity of liquids, has been utilised for many decades for measuring bacterial growth. In addition, it has found applications in the fields of brewing, water analysis, food and manufacturing chemistry (E.E.L. 1976), and has the advantage that measurements can be made without disturbing the test liquid.

Nephelometry works on the principle of light-scattering, so that light shining onto the test liquid becomes scattered if the liquid is turbid or cloudy. Connection of the nephelometer photo-cell to a galvanometer with linear scale allows detection and quantification of this light-scattering. Standards are used to set the galvanometer

scale to '0' and '100' (arbitrary units), so that comparison with other liquids can be made. In these experiments, deionised water was set as '0' on the galvanometer scale, and the standard ground perspex tube was set as '100'. All further measurements on the alginate/propranolol mixtures were made in comparison with these standards.

3.2.2 Materials

Laminaria hyperborea alginate, BN 902-282-04 (Protan Laboratories, Norway);

Laminaria hyperborea alginate (non-purified), BN 902-282 (Protan Laboratories, Norway);

Ascophyllum nodosum alginate, BN 911-211-04 (Protan Laboratories, Norway);

Propranolol hydrochloride BN 8905237 (CP Pharmaceuticals, Loughborough);

Sodium chloride BN 6794910K (BDH, Poole, Dorset);

EEL Unigalvo Type 200 Nephelometer Head/Galvanometer, E.E.L., Halstead, Essex.

3.2.3 Methods

A non-purified and a purified (section 3.1.4) alginate from *Laminaria hyperborea* (M:G ratio = 35:65) were used, together with an alginate from *Ascophyllum nodosum* (M:G ratio = 61:39, as characterised in Chapter 2). These alginates were chosen in order to investigate whether the purity of the alginate or its M:G ratio had an effect on the interaction with propranolol observed in the viscometry experiments.

Alginate (1gL^{-1}) and propranolol (0 to 0.05M) were mixed in various proportions and the turbidity measured in the nephelometer relative to the ground perspex standard. The effect on turbidity of adding different concentrations of sodium chloride to the propranolol/alginate mixtures was also investigated.

3.2.4 Results

Figures 3.5, 3.6 and 3.7 show the results for *L. hyperborea* (non-purified), *L. hyperborea* (purified) and *A. nodosum* alginates respectively, and the effect of adding sodium chloride to the propranolol/alginate mixtures on the concentration of propranolol at which precipitation occurs.

Figure 3.8 shows the effect of added sodium chloride on the visible precipitation point of the propranolol/alginate mixtures for the two purified alginates.

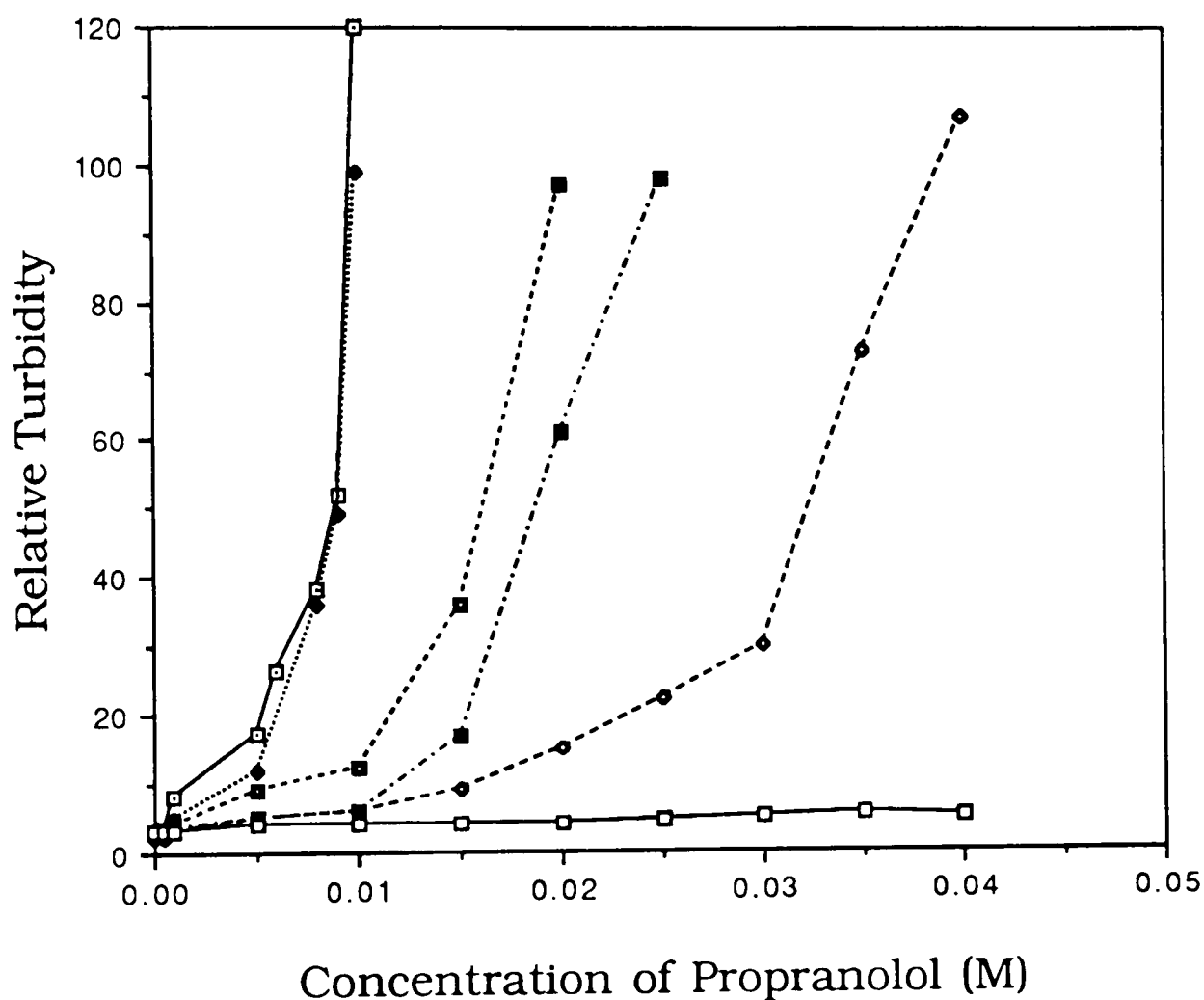
3.2.5 Discussion

Figures 3.5, 3.6 and 3.7 show that increasing the concentration of propranolol resulted in a marked increase in turbidity, which could be observed in the test mixtures as the appearance firstly of a cloudy suspension followed by the formation of floccules of a white-coloured precipitate as the concentration of propranolol was increased.

The precipitation point at each concentration of sodium chloride was found to be similar for both the non-purified and the highly-purified forms of *L. hyperborea* alginate, which would imply that the precipitate was not influenced by the interaction of propranolol with contaminants such as phenolic compounds in the alginate. In addition, little difference was observed in the precipitation point between the high-G *L. hyperborea* alginate and the high-M *A. nodosum* alginate. This is in marked contrast to the interaction of multivalent cations, such as calcium, which interact much more strongly with alginate G-blocks than with M-blocks (section 1.1.5).

From Figure 3.8, it can be seen that the addition of sodium as a counter-ion suppressed the formation of the precipitate. In addition, it was found that if a precipitate was first formed by the mixing of propranolol with alginate, this precipitate could be

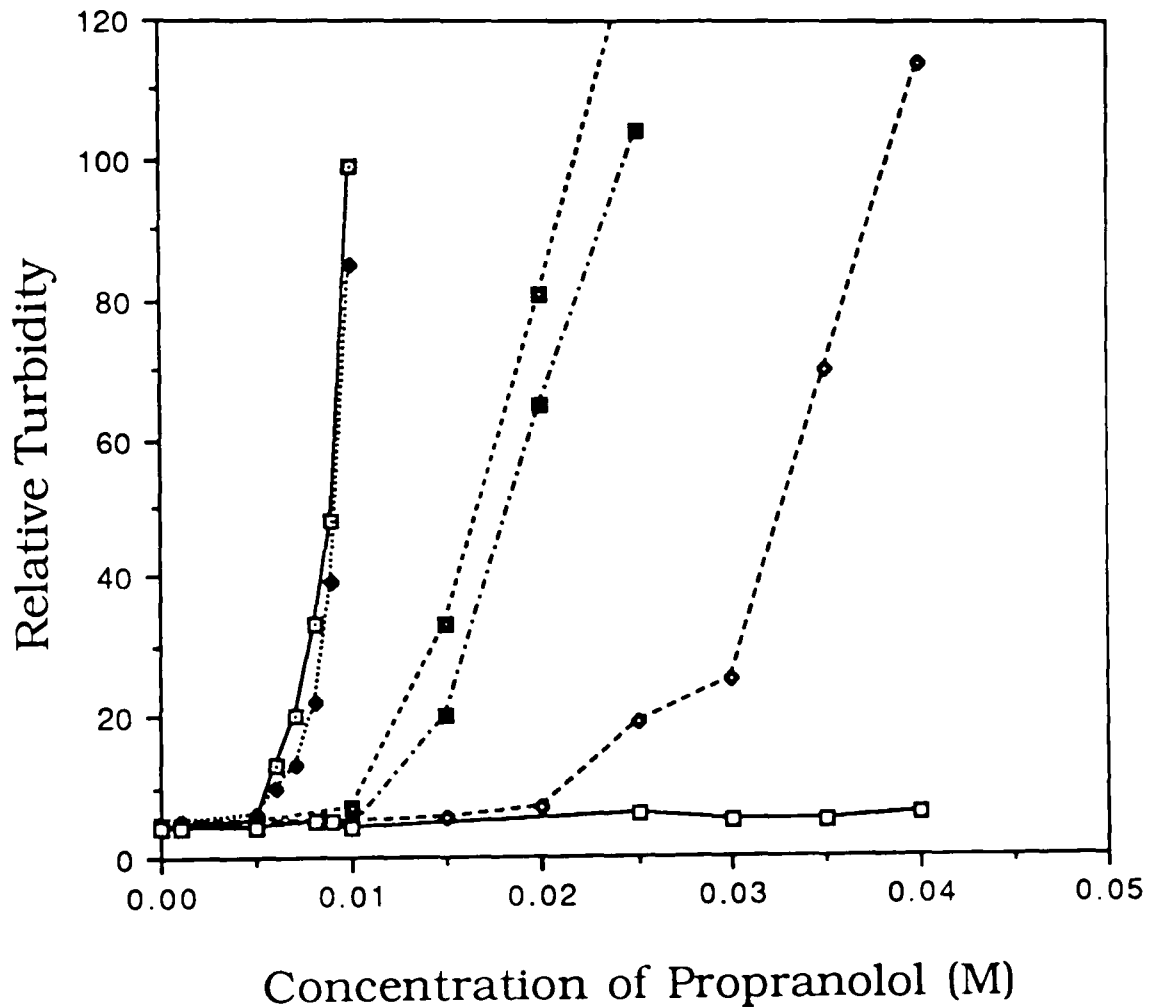
Figure 3.5 Nephelometry of Non-Purified *L. hyperborea* Alginate and Propranolol: The Effect of Sodium Chloride



Alginate Concentration = 1gL^{-1} in:

- Deionised water
-◆..... NaCl 0.01M
-■..... NaCl 0.1M
-◇..... NaCl 0.5M
-■..... NaCl 0.9%
- Control (Alginate conc. = 0)

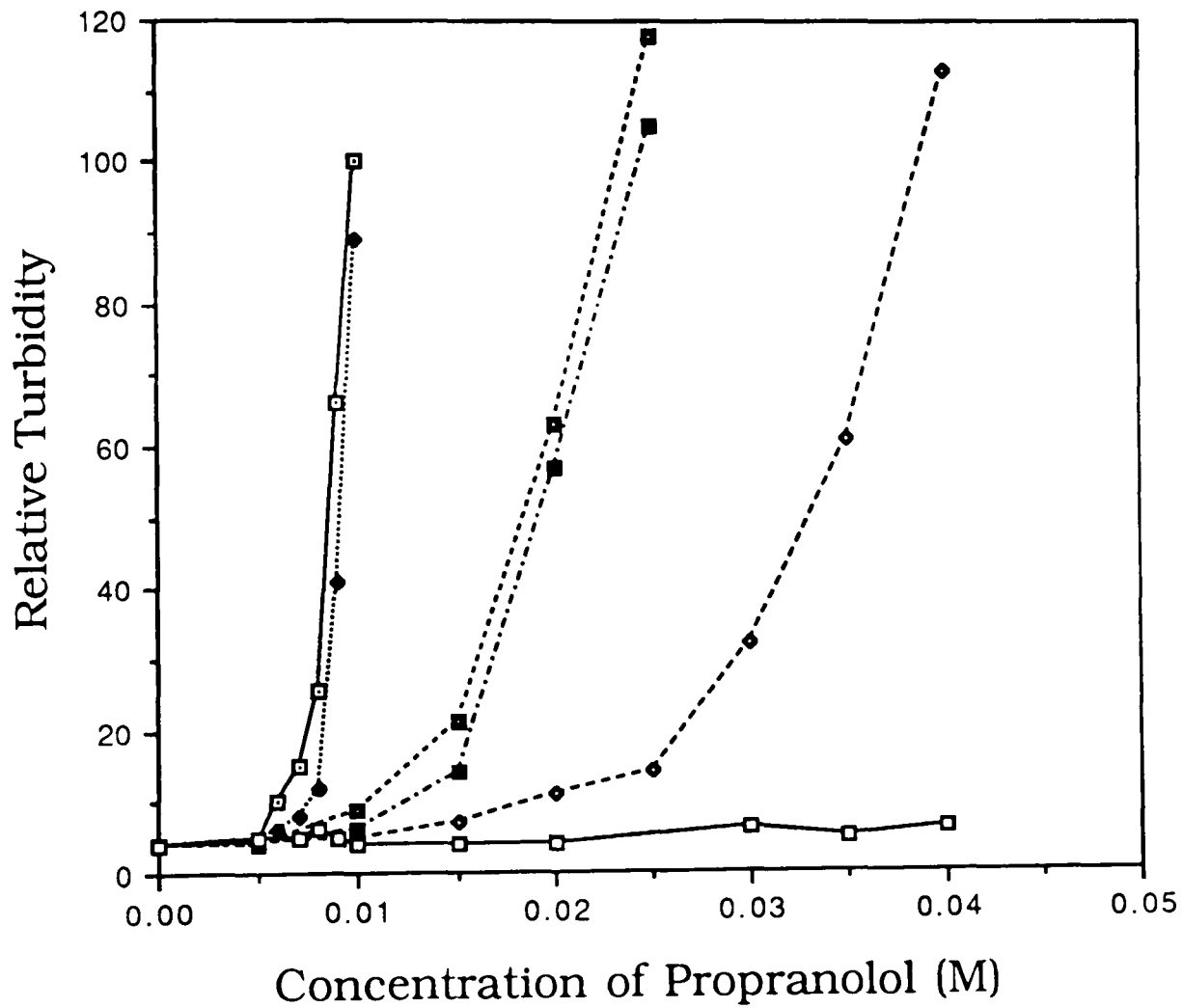
Figure 3.6 Nephelometry of Purified *L. hyperborea* Alginate and Propranolol: The Effect of Sodium Chloride



Alginate Concentration = 1gL^{-1} in:

- Deionised water
-◆..... NaCl 0.01M
-■..... NaCl 0.1M
-◇..... NaCl 0.5M
-■..... NaCl 0.9%
- Control (Alginate conc. = 0)

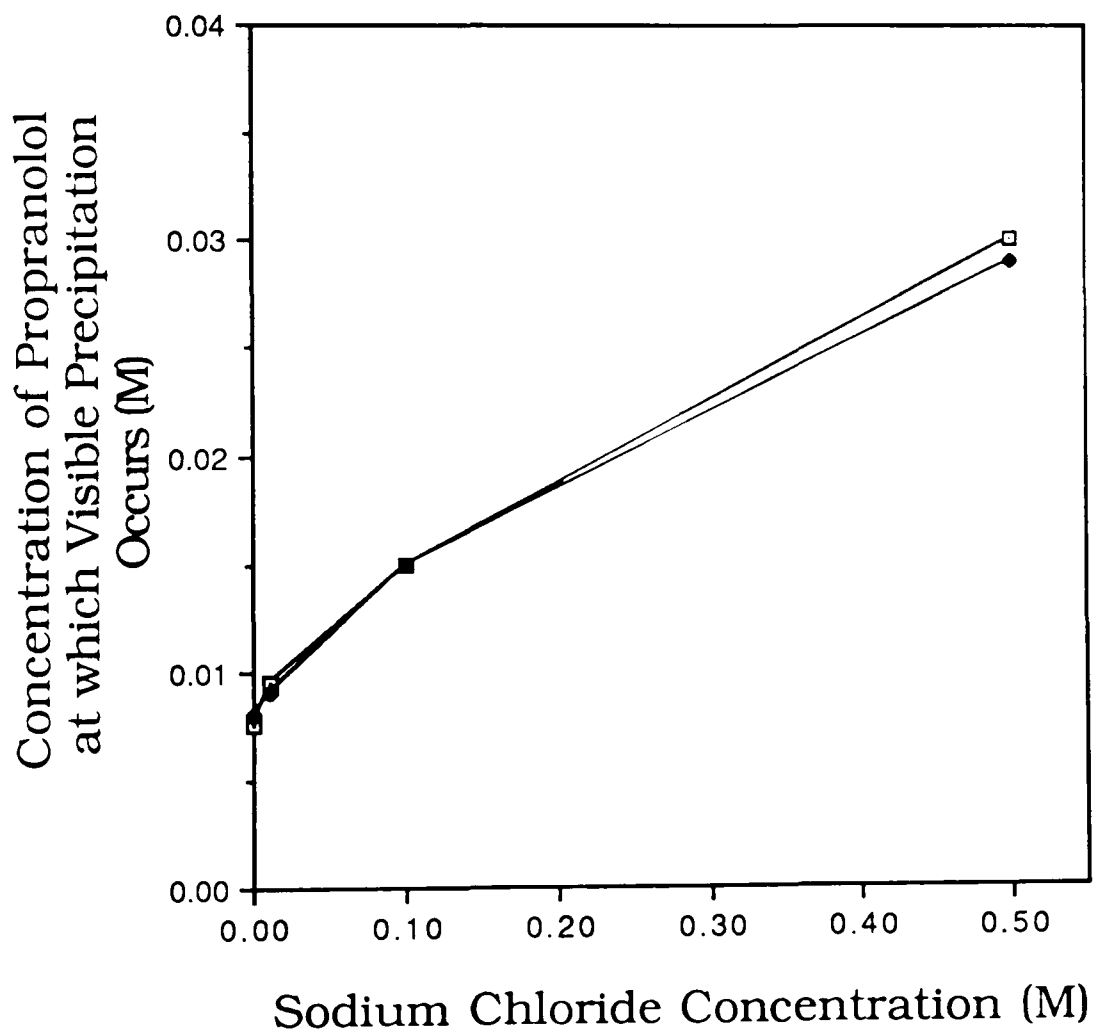
Figure 3.7 Nephelometry of Purified *A. nodosum* Alginate and Propranolol: The Effect of Sodium Chloride



Alginate Concentration = 1gL^{-1} in:

- Deionised water
-◆..... 0.01M NaCl
-■..... 0.1M NaCl
-◆..... 0.5M NaCl
-■..... 0.9% NaCl
- Control (Alginate conc. = 0)

Figure 3.8 The Effect of Sodium Chloride on the Visible Precipitation Point of Propranolol /Alginate Mixtures



—□— L. hyperborea
—●— A. nodosum

re-dissolved by the addition of sufficient sodium ions in the form of sodium chloride solution. The addition of a similar quantity of deionised water to the propranolol/alginate mixtures did not cause the precipitate to dissolve, therefore the possibility that the precipitate was dissolving merely by a dilution effect was ruled out.

Studies have been undertaken investigating the affinity of alginates for various cations (Smidsrød and Haug 1968). These have indicated that different ions have different affinities for binding to alginates, so that the addition of a counter-ion with a stronger binding affinity will result in the displacement of a weaker binding species from the alginate. From the experiments undertaken in the present studies, the solubilisation of the insoluble propranolol/alginate complex and the release of free propranolol on the addition of sodium counter-ions suggests that propranolol has a weaker affinity for alginate than sodium. This may be expected if one considers the relative molecular bulkiness of the two cations: propranolol would suffer greater steric hindrance than sodium ions when trying to bind to the alginate.

However, this system provides a potential mechanism for *in vivo* drug release from the propranolol/alginate complex by ion-exchange with simple cations in body fluids.

3.3 CONCLUSIONS

An interaction was observed between the alginates *L. hyperborea* or *A. nodosum* and the model drug propranolol hydrochloride, which resulted in the formation of a white-coloured precipitate. This precipitate could be re-dissolved by the addition of sodium (in the form of sodium chloride) as a counter-ion.

No difference in the point of precipitation was observed between the non-purified and purified forms of *L. hyperborea* alginate, or between the high-G *L. hyperborea* alginates and the high-M *A. nodosum* alginate.

In order to quantify the interaction between sodium alginate

and propranolol hydrochloride and to investigate the mechanism of complex formation, it is proposed to undertake binding studies on the purified *L. hyperborea* and *A. nodosum* alginates using equilibrium dialysis. These experiments will be described in Chapter 4.

CHAPTER 4 EQUILIBRIUM DIALYSIS **AND MOLECULAR MODELLING**

AIMS

Preliminary viscometry and nephelometry studies (Chapter 3) provided direct visual evidence that, above certain concentrations, there was an interaction between propranolol and sodium alginate in deionised water. The ability of added counter-ions (sodium chloride) to re-dissolve the white precipitate formed suggested that the interaction was ionic in nature, possibly a result of ion-pairing between the positively-charged drug (Figure 4.1) and the negatively-charged polysaccharide (Figure 4.2).

The objective in this chapter was to quantify this interaction in the presence and absence of sodium chloride, under carefully controlled conditions using an equilibrium dialysis technique. It was intended to investigate the stoichiometry of the interaction and to provide evidence of the type of binding from the shape of the binding isotherms. In addition, it was hoped that the formation of a soluble complex could be identified (in addition to the insoluble complex already observed) thereby providing an indication of the solubility limit of the propranolol/alginate complex. Before this work could be undertaken, the experimental procedure required validation in terms of non-specific binding, membrane preparation and integrity, and time to reach equilibrium for the system under study.

In addition, we attempted to gain further evidence for the nature of the binding by modelling the possible *in-vacuo* three-dimensional structure of the molecular complex using computational molecular modelling techniques.

Figure 4.1

Structure of Propranolol Hydrochloride

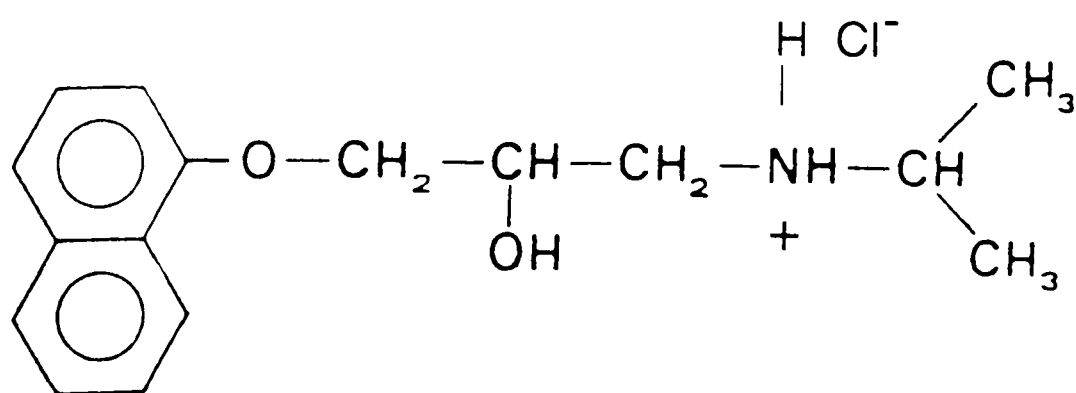
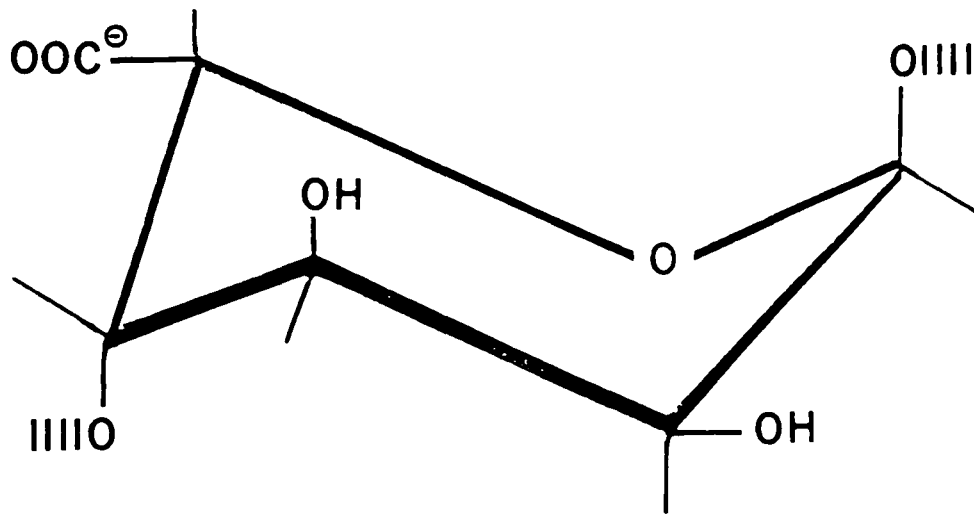


Figure 4.2

Structure of a Hexose Unit of Alginate



4.1 INTRODUCTION

4.1.1 Background

The phenomenon of dialysis was first described in 1861 by Graham (Graham 1861) and its subsequent evolution has been comprehensively reviewed by Carr (1961).

Equilibrium dialysis is an application of this general phenomenon which is extremely useful for gaining information about the extent of binding of ligands to macromolecules and is widely used because of its relative simplicity. It is one of several techniques available for investigating the interactions of small molecules with macromolecules, including, for example, gel permeation chromatography, sedimentation in the ultracentrifuge, and spectroscopic techniques including NMR.

Equilibrium dialysis was first applied to study the binding of low molecular weight compounds to biopolymers by Davis (1943). In 1946, Klotz used the technique to investigate the binding of azosulfonic acid with bovine serum albumin. The method, which was originally referred to as the 'knotted bag' method, has undergone considerable refinement since that time. Attention has focused on reducing the amount of time required to attain equilibrium, and on improving the reproducibility of results. For example, the recent availability of new and improved membranes has significantly speeded up the process, whilst the development of equipment such as the 'Dianorm' equilibrium dialyser (described by Weder et al 1971) enables experimental conditions to be standardised, thereby enhancing reproducibility.

In a typical equilibrium dialysis experiment, a solution of a macromolecule is separated from a solution of a low molecular weight compound (or ligand) by a semi-permeable membrane. All species present, except the macromolecule, are able to diffuse freely across the membrane. Equilibrium dialysis is based on the principle that, if unperturbed, the system will reach equilibrium such that the

chemical potentials of the ligand are equal on either side of the membrane. That is, the activity of the free ligand is equal on both sides of the membrane.

As a first approximation, provided dilute solutions are employed, the activity of the free ligand may be assumed to be equal to its concentration. Therefore, by measuring the final free ligand concentration, the amount of ligand bound to the macromolecule may be determined (see section 4.1.4).

4.1.2 The 'Dianorm' Apparatus

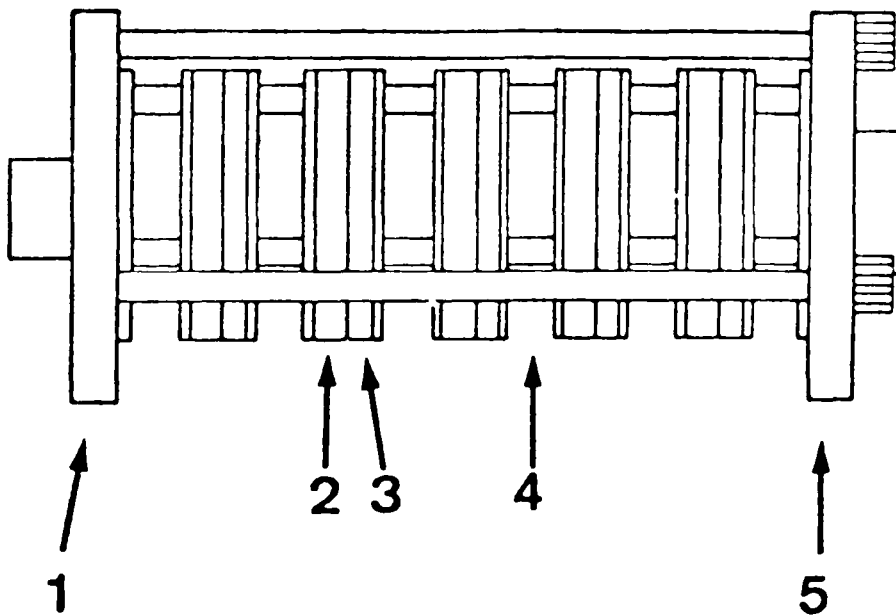
In the following binding experiments, the 'Dianorm' equilibrium dialysis system has been employed. The basic unit of the 'Dianorm' apparatus is a Teflon dialysis cell formed from two halves (the 'base' and the 'lid'), which are separated by a semi-permeable membrane. Each half contains three holes: one for sample application; the second acting as an air bleed; and the third for emptying of the sample. The cells are mounted in groups of five in a cell-carrier, each cell being separated by a spring-loaded spacer which acts as a sealing mechanism (Figure 4.3) Each group can then be attached to the carrier unit for rotation in a water bath at controlled temperature. The apparatus contains a total of twenty cells. The solution of drug is injected into one half-cell and the polymer solution into the other. The membrane is selected such that the drug can diffuse freely across it, whilst the polymer is completely retained.

The 'Dianorm' apparatus has been designed so that standardised reproducible equilibrium dialysis experiments can be performed for up to twenty samples simultaneously. The system has several other advantages over conventional bag dialysis:

(i) A high concentration gradient is maintained across the membrane, since mixing (by slow rotation at 4-20 r.p.m.) is continuous, therefore shorter dialysis times are required;

(ii) Smaller sample volumes can be used (approximately 3mL for the experiments described in section 4.3);

Figure 4.3
Diagrammatic Representation of a
'Dianorm' Cell Unit



1. Driving flange.
2. Teflon cell BASE)
Five pairs in complete stack
3. Teflon cell LID)
4. Spring loaded cell spacers (Six in complete stack).
5. Bearing flange secured with three knurled nuts.

(iii) The experiments may be carried out at any temperature between 0°C and 60°C. Rapid heat exchange between the water bath and the cell contents occurs because the cell walls are relatively thin (Weder et al 1971);

(iv) The filling and emptying of the cells is easily performed using Eppendorf/Gilson type pipettes;

(v) Recovery of samples after dialysis approaches 100%.

4.1.3. Membranes for Equilibrium Dialysis

It is a prerequisite that the membrane chosen for a particular equilibrium dialysis experiment must allow free passage of the ligand but not the macromolecule, and should not bind either the ligand or macromolecule to any appreciable extent. Compliance with these requirements must be ascertained prior to any dialysis experiments.

Transport of the ligand through the membrane obeys Fick's First Law of Diffusion such that the rate of diffusion depends upon the surface area of the membrane, the concentration gradient and the diffusion coefficient. (The diffusion coefficient is a function of temperature and a characteristic of the particular ligand molecule).

Membranes which are commonly used in equilibrium dialysis experiments include Visking, Spectrapor and Nucleopore. These vary in the material from which they are made (usually cellulose or polycarbonate), their permeability (related to pore size and membrane thickness), and their molecular weight cut-off points. Preference is normally given to membranes which fulfil the above requirements, but which give rise to the most rapid dialysis times.

4.1.4 General Theory of Binding

Consider the reversible binding of a ligand L to a macromolecule A:



with the equilibrium constant (K) for this association given by:

$$K = \frac{[AL]}{[A] \cdot [L]}$$

It is also convenient to refer to a 'dissociation constant' (K_d) which is merely the reciprocal of the above association constant:

$$K_d = \frac{[A] \cdot [L]}{[AL]} \quad \text{(Equation 4.1)}$$

The number of moles L bound per mole of A, under a given set of conditions may be defined as 'r' (the 'binding parameter'), where:

$$r = \frac{\text{concentration of L bound to A}}{\text{total concentration of all forms of A}}$$
$$r = \frac{[AL]}{[A] + [AL]}$$

From Equation 4.1:

$$[AL] = \frac{[A] \cdot [L]}{K_d}$$

So that:

$$r = \frac{([A][L]) / K_d}{[A] + ([A][L]) / K_d}$$

That is:

$$r = \frac{[L]}{K_d + [L]} \quad \text{(Equation 4.2)}$$

So far, only the case where one mole of a ligand (L) is bound by an individual site on a macromolecule (A) has been considered. However, it is possible that several moles of L may be bound by A.

Equation 4.2 (above) was derived for a single binding site. For the case where one mole of A can bind up to n moles of L, this equation is modified:

$$r = \frac{n [L]}{K_d + [L]} \quad \text{(Equation 4.3)}$$

where the n sites are assumed to be equal and independent (that is, the free energy of binding is the same for each site). K_d is now an average dissociation constant.

This fundamental equation can be rearranged into two main forms suitable for graphical treatment:

(i) By taking the reciprocal of each side of the equation:

$$\frac{1}{r} = \frac{1}{n} + \frac{K_d}{n [L]} \quad \text{(Equation 4.4)}$$

so that a plot of $1/[L]$ versus $1/r$ gives a straight line of slope K_d/n and an intercept on the y axis of $1/n$. This is known as the Double Reciprocal plot.

(ii) An alternative arrangement is:

$$\frac{r}{[L]} = \frac{n}{K_d} - \frac{r}{K_d} \quad \text{(Equation 4.5)}$$

so that a plot of r versus $r/[L]$ gives a straight line of slope $-1/K_d$ and an intercept on the x-axis of n . This is known as the Scatchard equation.

4.1.5 Analysis of Experimental Results from the 'Dianorm' Apparatus

The amount of time required to reach equilibrium is referred to as the 'dialysis time'. When this has elapsed, an equilibrium situation is attained so that the concentration of the free ligand ($C_{L(f)}$) is the same in both compartments of the cell. In the compartment containing no polymer, the free ligand concentration can be determined using a suitable analytical technique (in this case UV spectroscopy). The other compartment contains the bound plus the free ligand ($C_{L(f)} + C_{L(b)}$). Since the start concentration of the ligand ($C_{L(0)}$) and the concentration of the polymer (C_A) are known, the amount bound to the polymer can be easily calculated according to the following relationship:

$$C_{L(0)} = 2C_{L(f)} + C_{L(b)} \quad \text{(Equation 4.6)}$$

In some cases, it is found that the ligand binds to the

membrane or to the dialysis apparatus. However, this 'non-specific binding' can be quantified in a validation experiment and a correction factor introduced if necessary as follows:

$$C_{L(0)} = 2C_{L(f)} + C_{L(b)} + C_{L(m)} \quad \text{(Equation 4.7)}$$

where $C_{L(m)}$ is the concentration of ligand bound to the membrane or dialysis apparatus.

Using the nomenclature in section 4.1.4:

$$C_{L(b)} / C_A = r$$

and $C_{L(f)} = L$.

Thus, the experimentally determined values 'r' and ' $C_{L(f)}$ ' may be used to present the binding data as:

- (i) Binding isotherms, that is, plots of 'r' versus [L];
- (ii) Scatchard plots;
- (iii) Double Reciprocal plots.

These plots enable several parameters to be determined, including n, the number of binding sites on the alginate available for binding to propranolol. It is also possible to determine whether or not positive or negative co-operative binding is occurring (section 4.5).

4.2 MATERIALS AND EQUIPMENT

'Dianorm' equilibrium dialyser (Dianorm-Gerate, Germany);
Uvikon 860 UV Spectrophotometer, Kontron Instruments, England;
Visking dialysis tubing (cut-off point 5,000 Daltons);

Laminaria hyperborea alginate, BN 902-282-04 (Protan Laboratories, Norway);

Ascophyllum nodosum alginate, BN 911-211-04 (Protan Laboratories, Norway);

Propranolol hydrochloride BN 8905237 (CP Pharmaceuticals, Loughborough);

Sodium chloride BN 6794910K (BDH, Poole, Dorset);

AnaLar deionised water BN 3137510L (BDH, Poole, Dorset).

All were used as received.

4.3 VALIDATION EXPERIMENTS

Equilibrium dialysis techniques rely on the principle that the ligand under investigation will penetrate the membrane easily but that the macromolecule will be retained on one side of the membrane, and that the ligand will not bind significantly to the dialysis apparatus. Therefore, the following validation experiments were carried out prior to the binding experiments:

(i) suitability of the Visking membrane (verification of its ability to retain sodium alginate solution and to allow the free passage of propranolol hydrochloride);

(ii) determination of the time required to reach equilibrium for the propranolol/alginate system employed;

(iii) investigation of the non-specific binding of propranolol to the 'Dianorm' apparatus;

(iv) investigation of the non-specific binding of alginate to the 'Dianorm' apparatus.

4.3.1 Verification of the ability of the membrane to retain sodium alginate in a dialysis cell half

4.3.1.1 Methods

The following preparation procedure was undertaken prior to all experiments:

The membrane was cut into discs slightly larger than the dialysis cells. These were boiled for 30 minutes in deionised water, then soaked for three periods of 30 minutes in deionised water. After the third soak, the membranes were transferred to the experimental solution (either deionised water or sodium chloride) for two final soakings of 15 minutes each. Any residual solution was carefully removed with filter paper before placing the disc between the Teflon cells using plastic tweezers.

Prior to loading, the Teflon cells were cleaned thoroughly using a 5%v/v Decon solution and rinsed several times in deionised water, then dried at room temperature.

The dialysis cells were filled as follows:

Test cells:

BASE : 3mL sodium alginate solution (2gL^{-1} or 4gL^{-1} in deionised water).

LID : 3mL deionised water.

Control cells:

BASE : 3mL deionised water.

LIDS : 3mL deionised water.

Temperature: 25.0°C .

Dialysis time: 72 hours, 20 rotations per minute.

Membrane: Visking, cut-off point (nominal) 5000 Daltons.

After dialysis, a 1mL sample from the lids of the dialysis cells was subjected to the phenol-sulphuric acid test (Dubois et al 1956) as described in section 2.2.5.1.

4.3.1.2 Results

Tables 4.1 and 4.2 show the results after subjecting the membrane to the preparation procedure described above (4.3.1.1).

Table 4.1 Verification of the ability of the semi-permeable membrane to retain sodium alginate in a dialysis cell half

Laminaria hyperborea alginate 2gL⁻¹

<u>TEST CELLS</u>		<u>CONTROL CELLS</u>	
Sample	UV Abs. (480nm)	Sample	UV Abs. (480nm)
1	0.021	1	0.005
2	0.036	2	0.009
3	0.012	3	0.027
4	0.014	4	0.032
5	0.039	5	0.034
6	0.015	6	0.022
7	0.022	7	0.033
8	0.026	8	0.016
9	0.008	9	0.007
10	0.014	10	0.013
Mean = 0.021		Mean = 0.020	
SD = 0.009		SD = 0.01	

Table 4.2 Verification of the ability of the semi-permeable membrane to retain sodium alginate in a dialysis cell half

Laminaria hyperborea alginate 4gL⁻¹

<u>TEST CELLS</u>		<u>CONTROL CELLS</u>	
Sample	UV Abs. (480nm)	Sample	UV Abs. (480nm)
1	0.031	1	0.028
2	0.027	2	0.031
3	0.009	3	0.017
4	0.013	4	0.009
5	0.016	5	0.007
6	0.033	6	0.018
7	0.026	7	0.030
8	0.021	8	0.016
9	0.015	9	0.010
10	0.020	10	0.029
Mean = 0.021		Mean = 0.0195	
SD = 0.008		SD = 0.009	

The results for the alginate test cells compare favourably with the control cells containing only deionised water. This indicates that *Laminaria hyperborea* alginate is retained by the membrane in one dialysis half-cell and gives confidence in the preparation and cell-assembling procedure, in that the membrane appears to remain intact.

A small amount of saccharide was detected in both the control cells and in the test cells by the phenol-sulphuric acid test. However, this test is not specific for uronic acids, but is a general test for simple sugars, oligosaccharides and polysaccharides. It is

therefore possible that this was a result of material leaching from the cellulose membrane into both the control and the test solutions, as the effect was found to be greatly reduced by washing the membrane. The membrane preparation procedure described above was therefore employed in all future experiments.

4.3.2 Investigation of the time required to reach equilibrium for a propranolol/alginate system and verification of the passage of propranolol across the semi-permeable Visking membrane

The dialysis time must be determined for each individual ligand which will be used in dialysis experiments, in order to ensure that the system has reached equilibrium before the cell contents are analysed. Thus, for the purpose of these experiments, it was necessary to determine the dialysis time for propranolol hydrochloride (MW = 295.8 Daltons) in the presence of *Laminaria hyperborea* alginate.

4.3.2.1 Methods

The dialysis cells were filled as follows:

BASE : 3mL propranolol solution (0.01M or 0.1M)

LID : 3mL deionised water or alginate solution 1gL^{-1} .

Temperature: 25.0°C.

Dialysis time: 8 hours, 20 rotations per minute.

Membrane: Visking, cut-off point (nominal) 5000 Daltons.

Dialysis of a known concentration of propranolol against deionised water was performed under the same conditions as those proposed for the subsequent binding experiments (that is, temperature 25.0°C; 20 rotations per minute; Visking membrane with cut-off point at approximately 5000 Daltons). The change in concentration of propranolol in both half-cells was determined as a function of time until equilibrium was attained. Propranolol

concentration was determined spectrophotometrically using a Kontron Uvikon UV spectrophotometer at wavelength 288nm.

These experiments were repeated in the presence of *Laminaria hyperborea* alginate in order to ensure that the presence of the alginate did not significantly increase the time to reach equilibrium.

4.3.2.2 Results

Figures 4.4 and 4.5 show the results for propranolol concentrations of 0.1 M and 0.01M respectively. Equilibrium is reached at the point where the two curves asymptote the line at approximately 50% of the starting concentration. Figure 4.6 shows the results for propranolol 0.1M in the presence of alginate 1.0gL⁻¹.

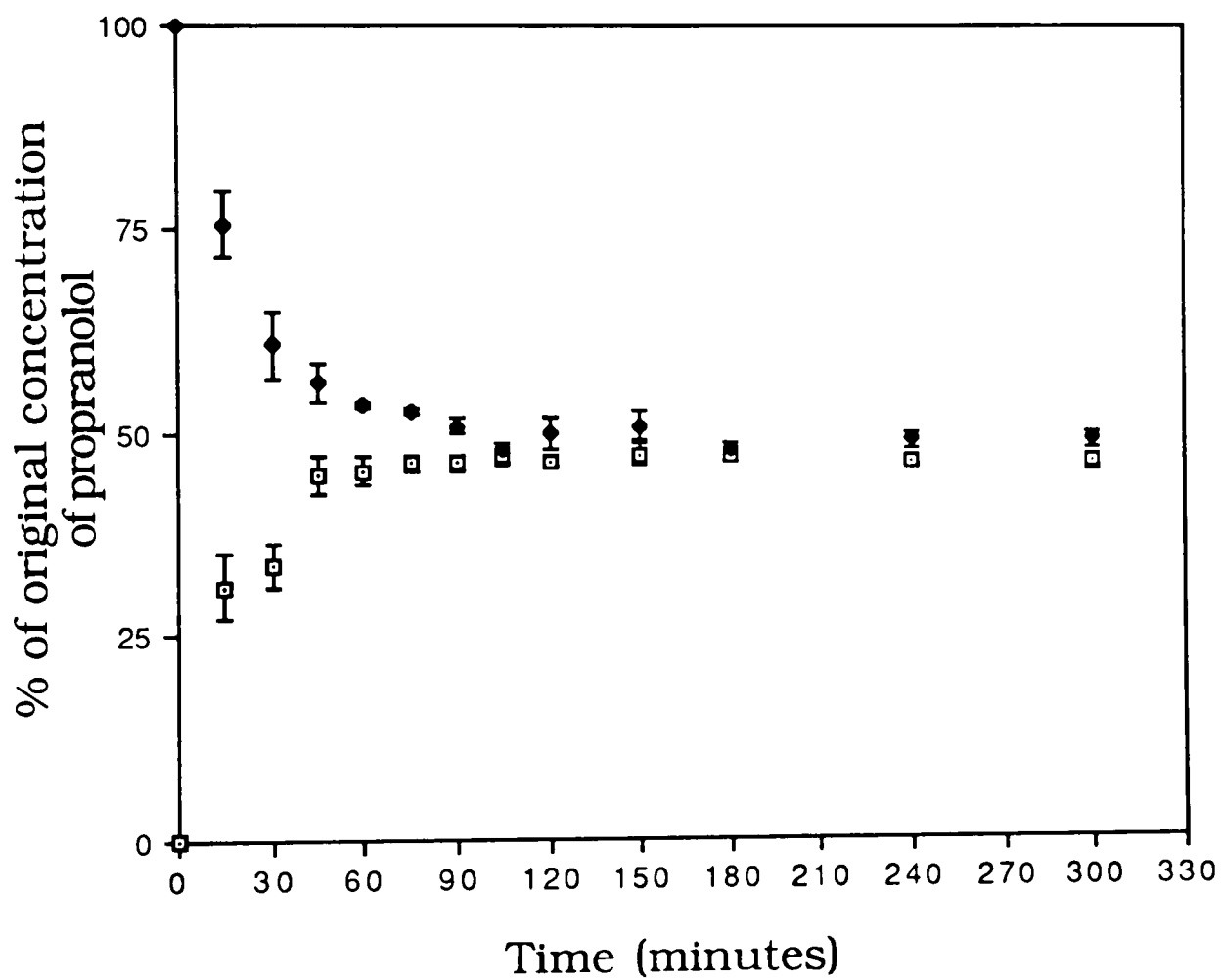
Thus, in each case, under the conditions outlined above, equilibrium was attained in approximately 3-4 hours. In subsequent experiments the dialysis time was set at 5 hours or greater to ensure that equilibrium had been reached.

These results also demonstrate that propranolol hydrochloride (MW = 295.8 Daltons) passes freely through the proposed dialysis membrane (cut-off point 5000 Daltons), as expected.

4.3.3. Investigation of non-specific binding of propranolol hydrochloride to the Dianorm equilibrium dialysis apparatus

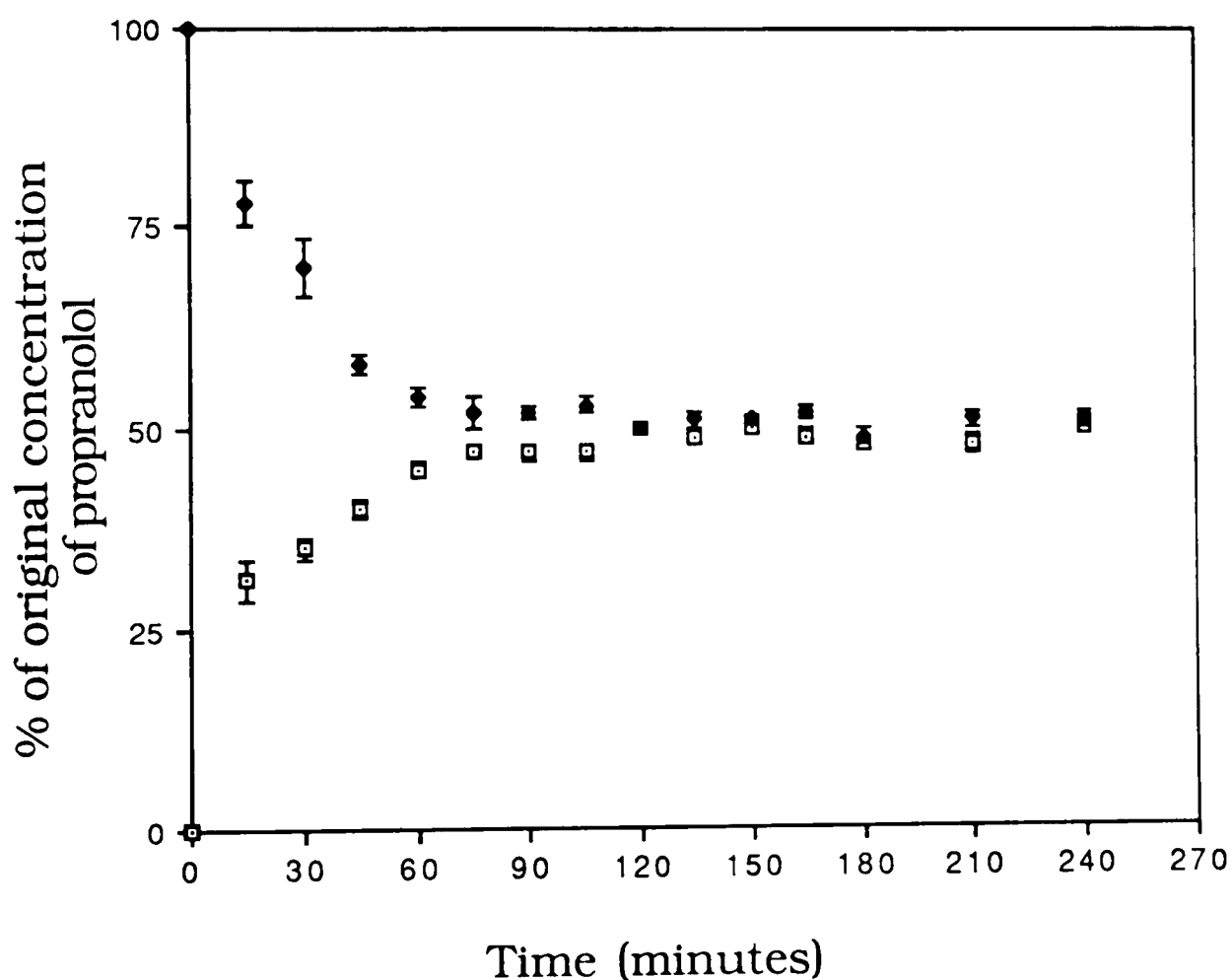
In any equilibrium dialysis experiment, there is a possibility that the experimental ligand may bind to the membrane or the apparatus, resulting in a decrease in available free ligand. Therefore, it is necessary to quantify this 'non-specific' binding before proceeding with the actual equilibrium dialysis experiments. Depending on the degree of non-specific binding, it may either be considered negligible in comparison to experimental binding, or require a correction to be made to the results of binding experiments.

Figure 4.4 Determination of Time to Reach Equilibrium in the 'Dianorm' Equilibrium Dialyser. Propranolol Concentration 0.1M



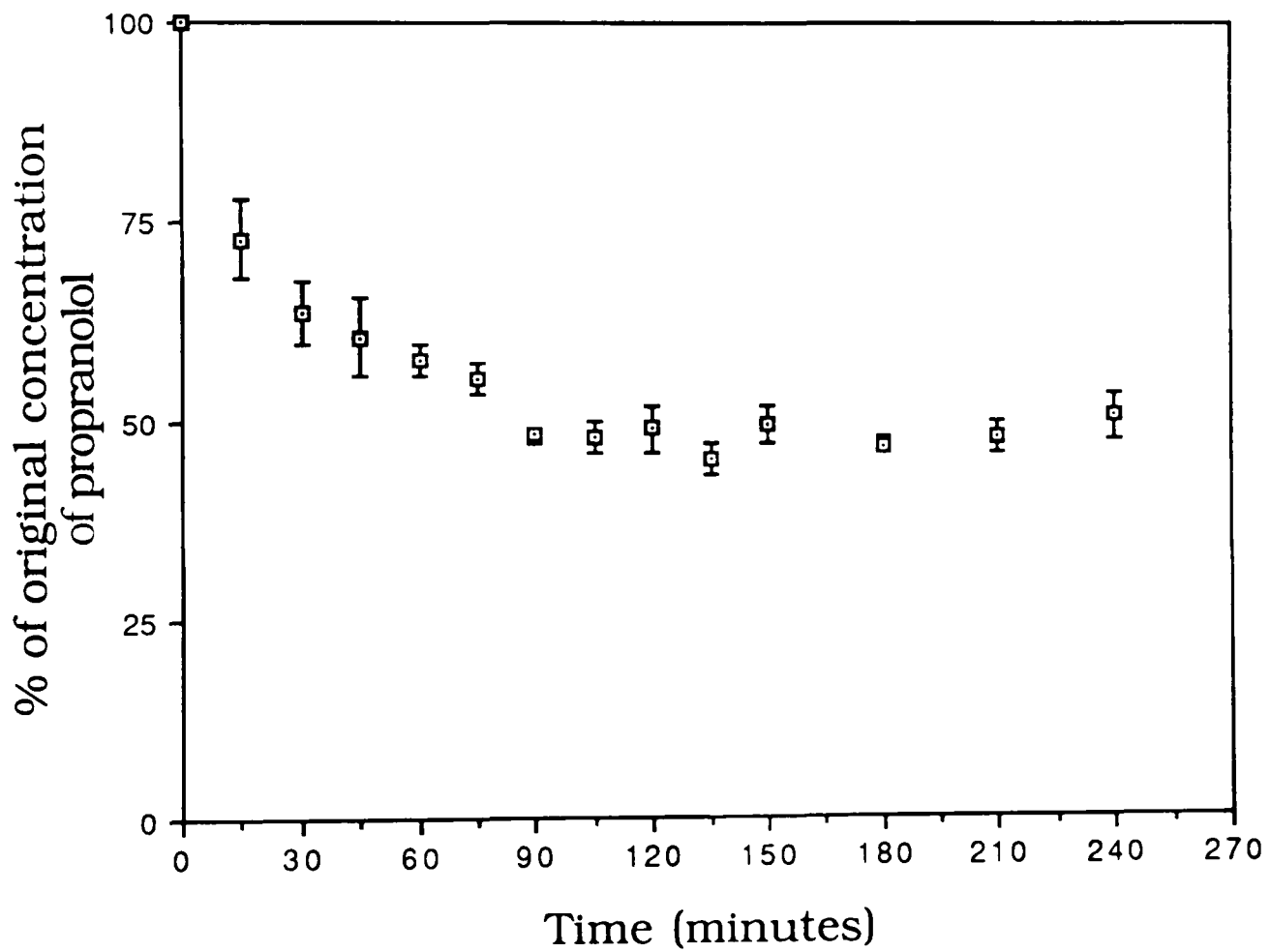
- Concentration of propranolol in donor compartment
- Concentration of propranolol in acceptor compartment

Figure 4.5 Determination of Time to Reach Equilibrium in the 'Dianorm' Equilibrium Dialyser. Propranolol Concentration 0.01M



- Concentration of propranolol in donor compartment
- Concentration of propranolol in acceptor compartment

Figure 4.6 Determination of Time to Reach Equilibrium in the 'Dianorm' Equilibrium Dialyser. Alginate 1gL⁻¹ ; Propranolol 0.1M.



□ Concentration of propranolol in donor compartment

4.3.3.1 Methods

The dialysis cells were filled as follows (five samples for each concentration of propranolol):

BASE : 3mL propranolol solution (0.001M, 0.01M, 0.1M or 0.5M)

LID : 3mL deionised water.

Temperature: 25.0°C.

Dialysis time: 24 hours, 20 rotations per minute.

Membrane: Visking, cut-off point (nominal) 5000 Daltons.

After dialysis, 2mL samples from both compartments of each sample cell were analysed at 288nm in a Kontron Uvikon UV spectrophotometer for the presence of propranolol. Any loss of propranolol during the dialysis experiments was assumed to be due to non-specific binding to the 'Dianorm' apparatus.

4.3.3.2 Results

Table 4.3 shows the results obtained.

Table 4.3 Investigation of non-specific binding of propranolol to the 'Dianorm' apparatus.

<u>Start Conc.</u> <u>Propranolol</u> <u>(M)</u>	<u>Final Conc.</u> <u>Propranolol</u> <u>in Base (M)</u>	<u>Final Conc.</u> <u>Propranolol</u> <u>in Lid (M)</u>	<u>Loss of</u> <u>Propranolol</u> <u>due to Non-</u> <u>Specific</u> <u>Binding (%)</u>
0.001	5×10^{-4}	4.9×10^{-4}	1
0.01	0.0050	0.0050	0
0.1	0.0495	0.0500	0.5
0.5	0.2500	0.2470	0.6

These results indicate that over a wide concentration range the degree of non-specific binding is negligible ($\leq 1\%$) in comparison to the amount of available free propranolol. Thus, for these experiments, there was no need for a correction factor to be applied when determining binding constants for propranolol/alginate systems at these concentrations.

4.3.4. Investigation of non-specific binding of sodium alginate to the Dianorm equilibrium dialysis apparatus

As for the previous experiments to determine the non-specific binding of propranolol to the Dianorm apparatus (4.3.3) it was also necessary to quantify any non-specific binding of sodium alginate.

4.3.4.1. Methods

The dialysis cells were filled as follows:

BASE : 3mL sodium alginate solution (1gL^{-1} , 2gL^{-1} or 4gL^{-1} in deionised water).

LID : 3mL deionised water.

Temperature: 25.0°C .

Dialysis time: 72 hours, 20 rotations per minute.

Membrane: Visking, cut-off point (nominal) 5000 Daltons.

After dialysis, a 1mL sample from both compartments of the dialysis cells was subjected to the phenol-sulphuric acid test (Dubois et al 1956) as described in section 2.2.5.1. Any loss of alginate during the dialysis experiments was assumed to be due to non-specific binding to the Dianorm apparatus.

4.3.4.2 Results

Table 4.4 shows the results obtained.

Table 4.4 Investigation of non-specific binding of sodium alginate to the 'Dianorm' apparatus.

<u>Start Conc.</u> <u>Alginate</u> <u>(gL⁻¹)</u>	<u>Final Conc.</u> <u>Alginate</u> <u>in Base (gL⁻¹)</u>	<u>Final Conc.</u> <u>Alginate</u> <u>in Lid (gL⁻¹)</u>	<u>Loss of</u> <u>Alginate</u> <u>due to Non-</u> <u>Specific</u> <u>Binding (%)</u>
1.0	0.99	0.01	0
2.0	1.97	0.01	1
4.0	3.97	0.02	0.25

These results indicate that, over a range of concentrations, the amount of non-specific binding of alginate was negligible ($\leq 1\%$). Thus, for these experiments, there was no need for a correction factor to be applied when determining binding constants for propranolol/alginate systems at these concentrations.

4.3.5 Conclusions

The time required to reach equilibrium using the 'Dianorm' equilibrium dialysis apparatus for a propranolol and a propranolol/alginate system was of the order of 3-4 hours.

The membrane chosen (Visking, nominal cut-off point 5000 Daltons) was found to be suitable for the propranolol/alginate system to be studied since it allowed free passage of propranolol hydrochloride solution, but retained sodium alginate solution in a dialysis cell-half.

The 'Dianorm' apparatus was found to be suitable for the propranolol/alginate system to be studied, since the non-specific binding of propranolol hydrochloride in the 'Dianorm' apparatus was found to be negligible.

4.4 METHODS

4.4.1 Binding experiments

The dialysis membrane used in these experiments was Visking tubing with a cut-off point of 5000 Daltons. The validation experiments (section 4.3) showed that preparation of the membrane was essential to eliminate adherent impurities which could interfere with the subsequent binding experiments. The preparation procedure described in section 4.3.2.2 above was therefore undertaken prior to all experiments.

The dialysis cells were assembled by sandwiching a disc of pre-treated semi-permeable membrane (see section 4.3.3) between two half-cells. Five cells were then mounted in a cell-carrier, and four assembled carriers mounted in a rotating drive unit, in a water bath at $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

The cells, each with a half-cell volume of 5mL, were filled with 3.0mL of propranolol hydrochloride solution in one half, and 3.0mL of alginate solution in the other, using a Gilson pipette. The 2mL void space ensures adequate mixing of the solution and good membrane contact for the duration of the experiment.

After each dialysis run, the solution on the alginate-free side of the cell was drained, and the concentration of free propranolol determined spectrophotometrically at 288nm.

The following experiments were carried out:

- (i) The binding of propranolol to *Laminaria hyperborea* alginate (1gL^{-1});
- (ii) The binding of propranolol to *Ascophyllum nodosum* alginate (1gL^{-1})
- (iii) The effect of sodium chloride on the binding of propranolol to each alginate.

4.4.2 Molecular Modelling

The possible *in vacuo* three-dimensional structure of a propranolol/alginate complex was investigated using the 'COSMIC' (COmputation and Structure Manipulation In Chemistry) Molecular Modelling System (Vinter et al 1987). This is a molecular graphics and computational chemistry framework which has been designed primarily to handle small molecules of up to 300 atoms and which has evolved as a molecular modelling aid for use in the pharmaceutical industry.

The propranolol and alginate molecules were first sketched freehand using the 'COSMIC' system, then the charges on each of the atoms were calculated using the programme 'Charge 2' (Abraham and Smith 1988). The two molecules were then allowed to dock together using the 'single hard sphere' docking method described by Vinter et al (1987). The 'Cosmic Force Field' programme (Morley et al 1991) (which calculates the energy of a molecule given a certain geometry) was used to 'minimise' the structure, that is, to calculate the structure which has the lowest energy.

In summary, the 'stationary' molecule is placed at the centre of a 10Å sphere and the 'mobile' molecule is placed in turn at points on the sphere about 1Å apart, a total of 204 starting points. At each starting point, the mobile molecule is rotated about its centre in 60° steps about all three axes, and the lowest energy obtained defines the starting orientation for a subsequent simplex energy minimisation. Each molecule is treated as a rigid body, and no relaxation of bond lengths, angles or torsional angles is allowed. The simplex runs for a maximum of 500 iterations, or until the change in energy between successive iterations is less than 0.0001 kcal/mole. All 204 final positions of the mobile molecule are stored by the computer. The photographs obtained show the lowest energy complex of the 204 that are produced by the docking routine described above.

4.5 RESULTS

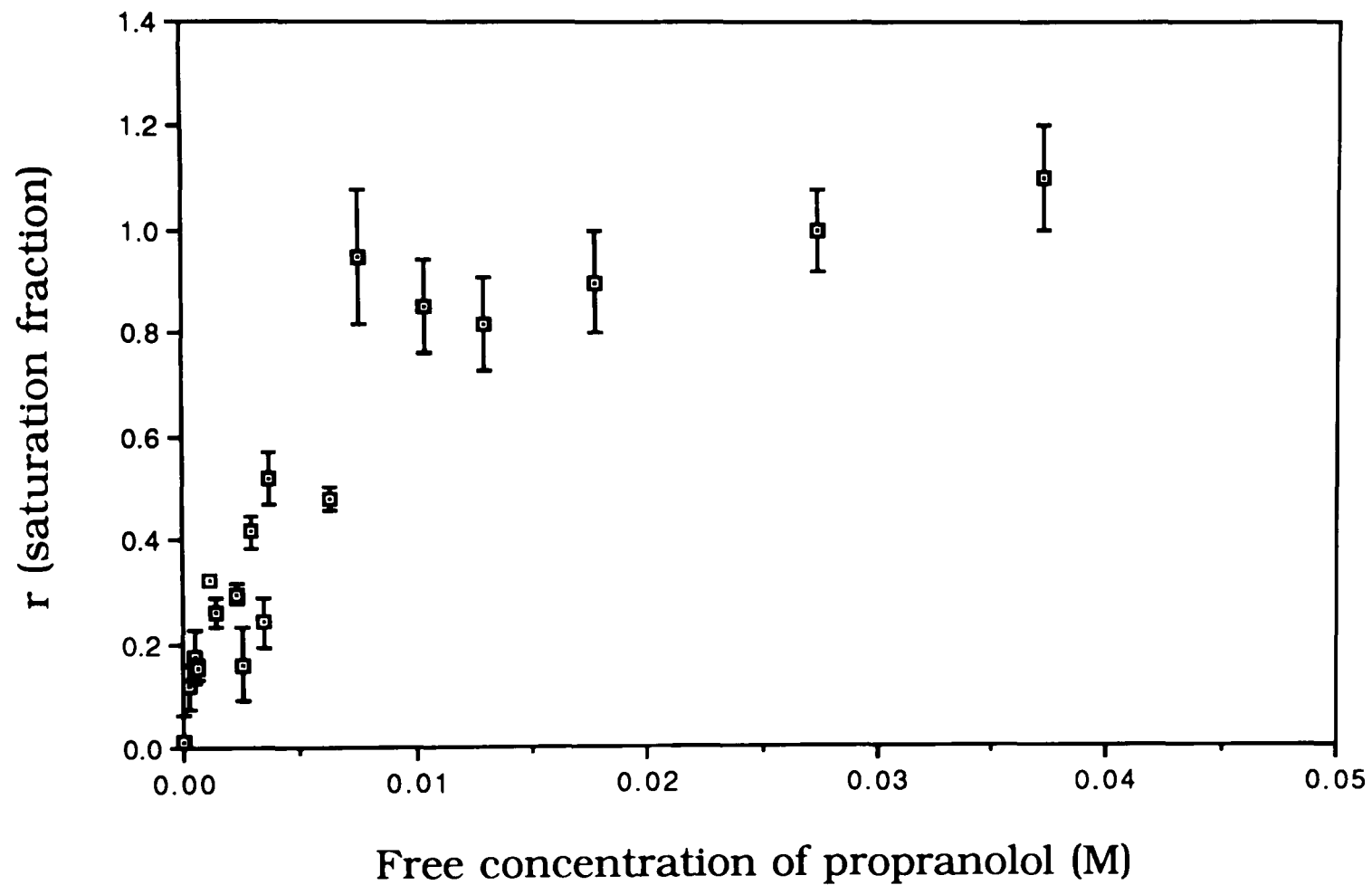
Figures 4.7 and 4.8 show the binding isotherm ($C_{L(f)}$ vs. r) for the binding of propranolol to *L. hyperborea* alginate and *A. nodosum* alginate respectively in distilled water.

The Scatchard equation and the Double Reciprocal equation (section 4.1.4) were used to determine the binding parameters discussed in section 4.1.4 above. Figures 4.9 and 4.10 show a Scatchard plot and a Double Reciprocal plot for *L. hyperborea* alginate, and Figures 4.11 and 4.12 show the same plots for *A. nodosum* alginate. (Note that the curves through the points for Figures 4.9 to 4.12 were fitted by eye and are therefore for guidance only, in order to show the general trend of the plots).

Figures 4.13 and 4.14 illustrate the influence of sodium chloride on the binding of propranolol (0.001M and 0.02M respectively) to the two alginates (1gL^{-1}).

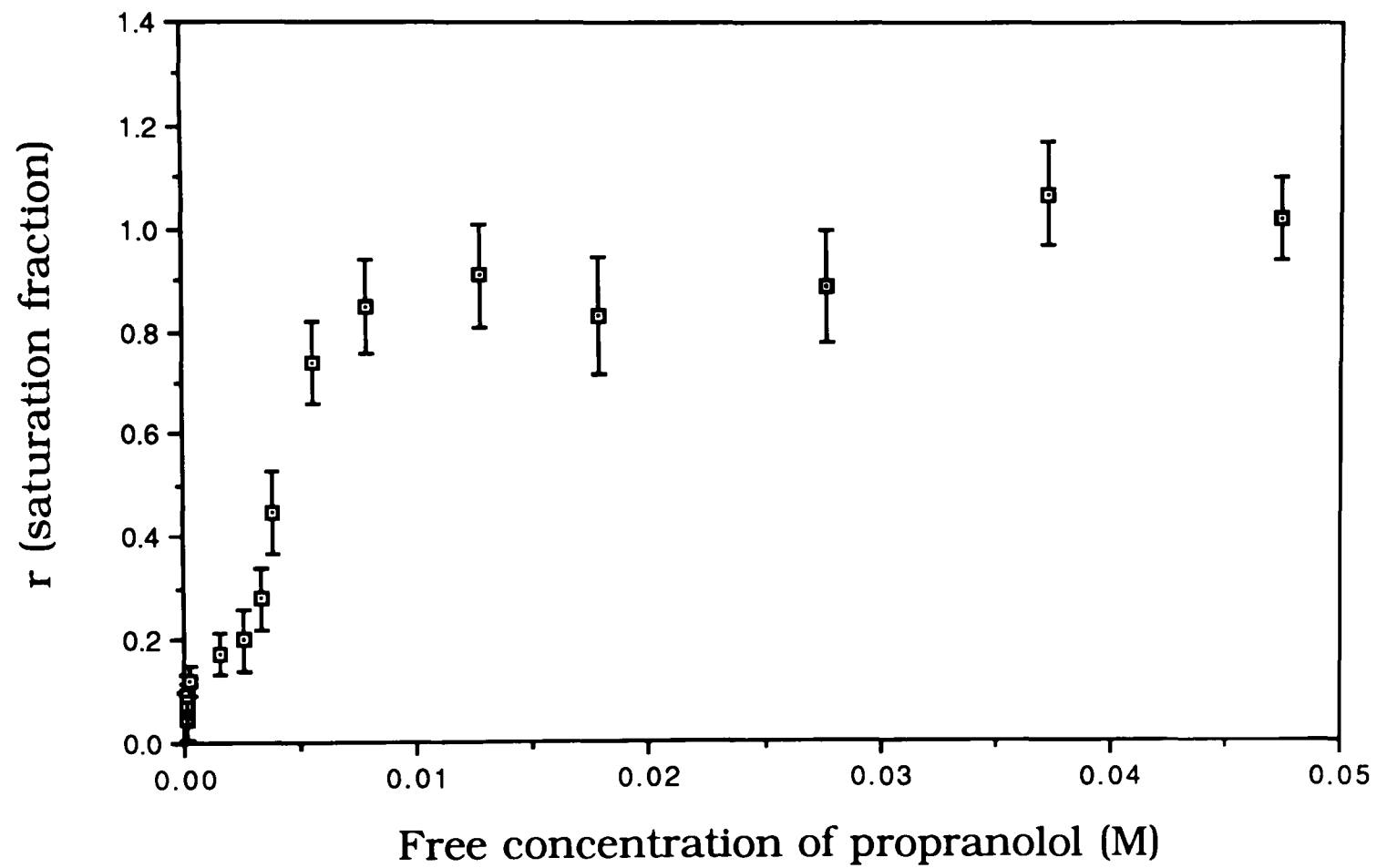
Figure 4.15 shows the possible *in vacuo* three-dimensional structure of the propranolol/alginate complex as modelled using the 'COSMIC' Molecular Modelling System. (Oxygen is represented in red and nitrogen in blue).

Figure 4.7. Binding Isotherm for the Binding of Propranolol to *L. hyperborea* Alginate



Each Point:
Mean (n = 10-15) \pm 1SD
Alginate Conc = 1gL⁻¹

Figure 4.8 Binding Isotherm for the Binding of Propranolol to *A. nodosum* Alginate



Each point:
Mean (n = 10-15) \pm 1SD
Alginate Conc = 1gL⁻¹

Figure 4.9 Binding of Propranolol to L. hyperborea Alginate - Scatchard Plot

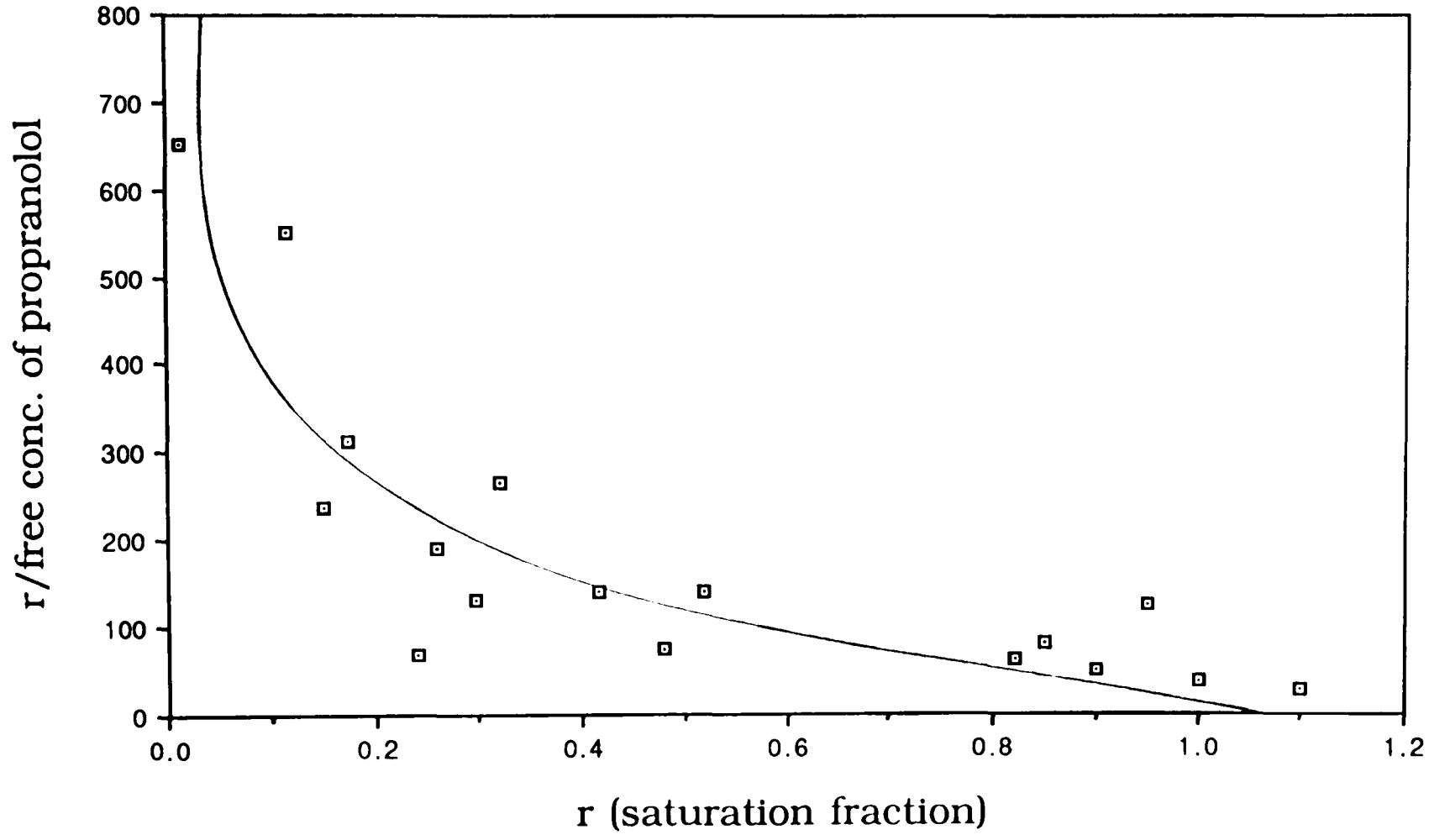


Figure 4.10 Binding of Propranolol to
L. hyperborea Alginate - Double
Reciprocal Plot

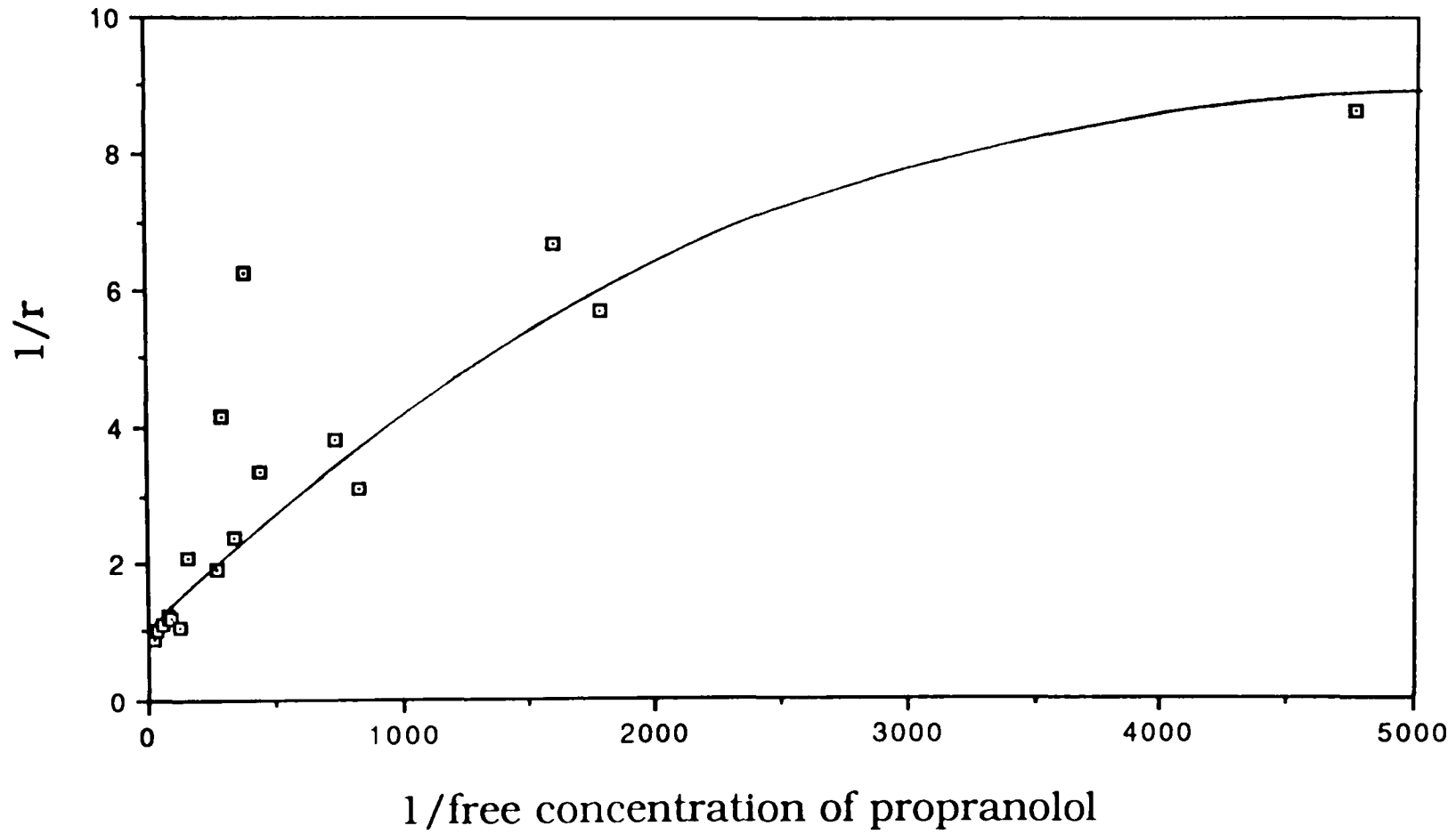


Figure 4.11 Binding of Propranolol to A. nodosum Alginate - Scatchard Plot

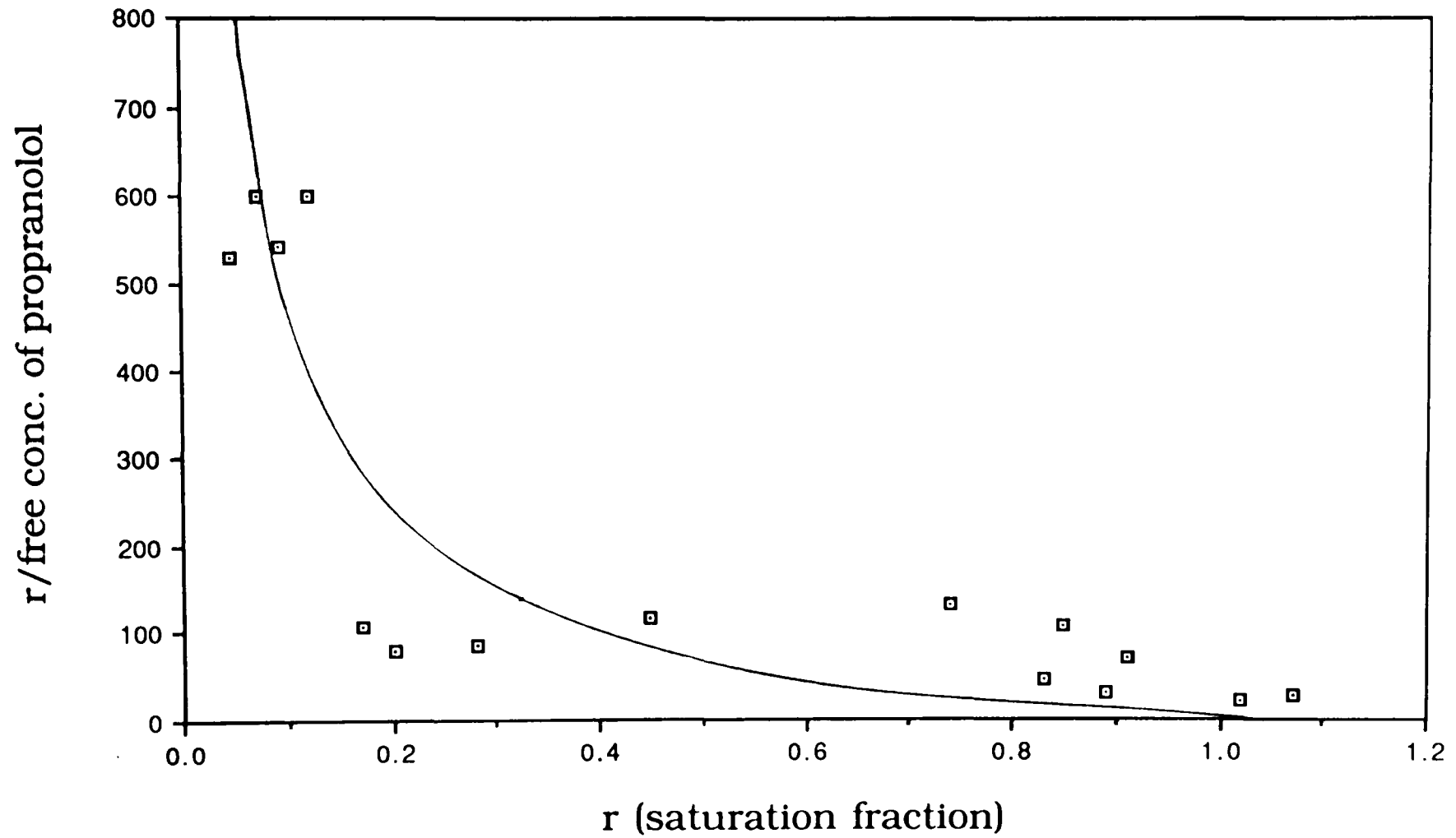


Figure 4.12 Binding of Propranolol to
A. nodosum Alginate - Double
Reciprocal Plot

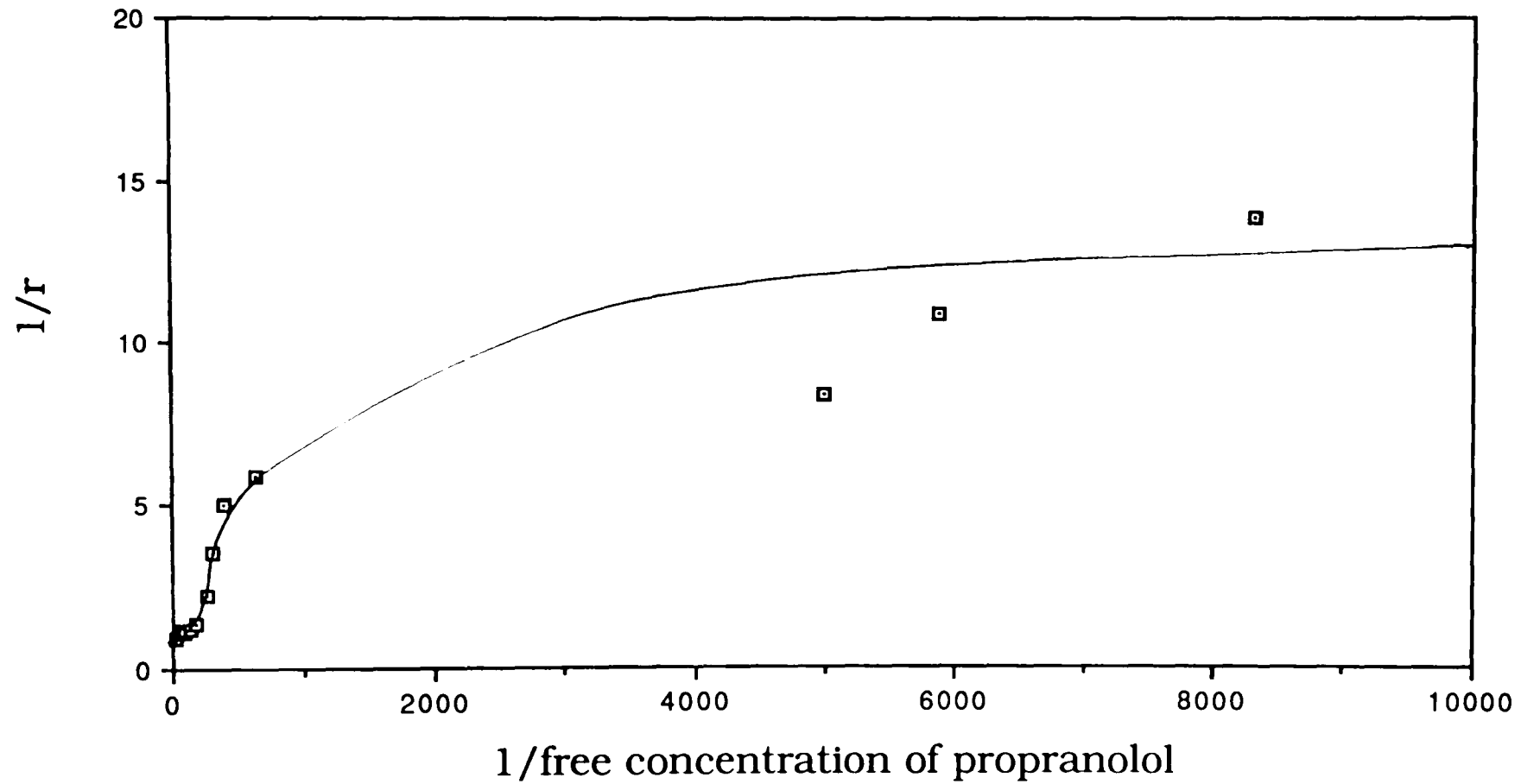
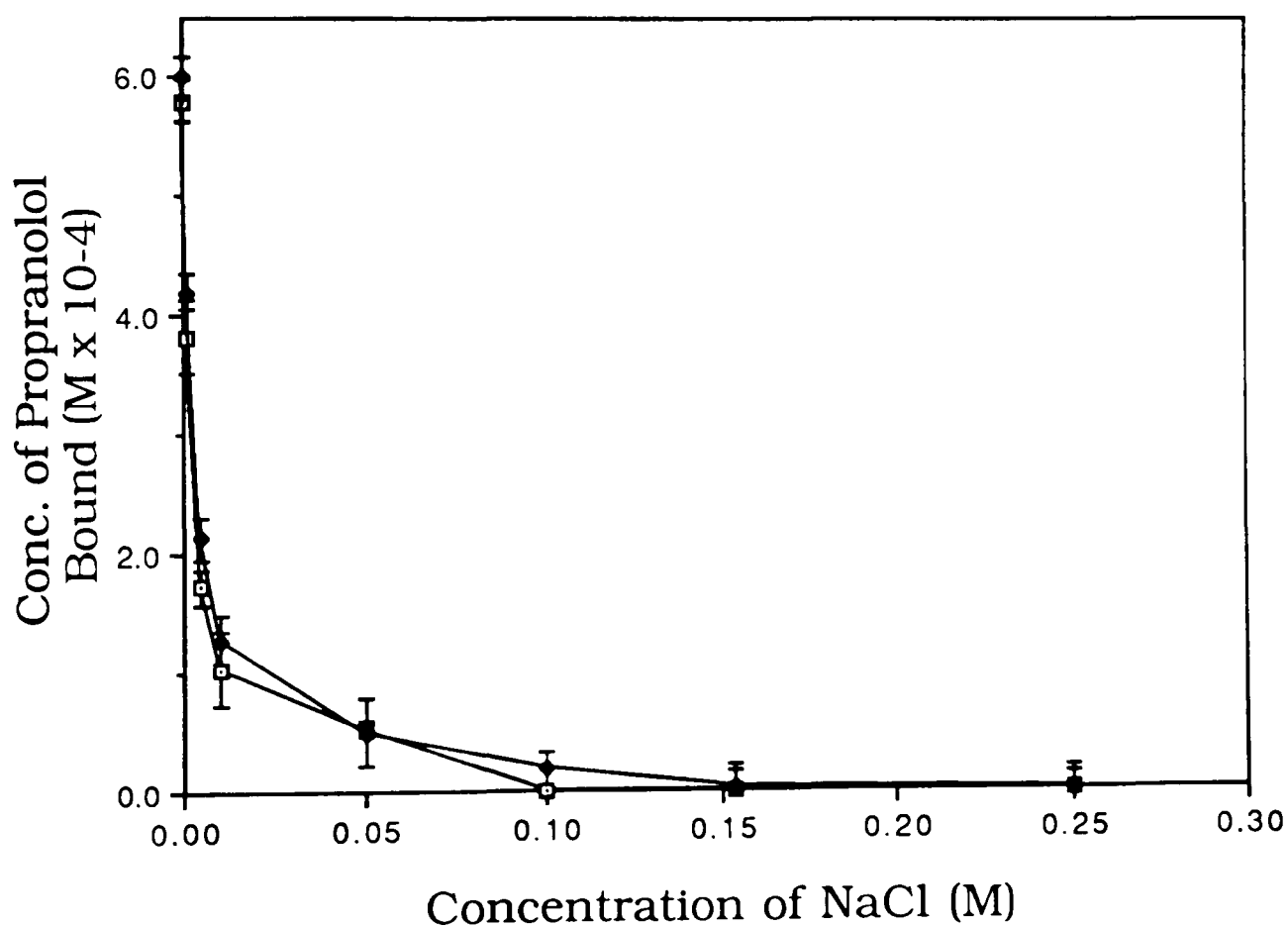


Figure 4.13 The Effect of Added Sodium Chloride on the Binding of Propranolol to *L. hyperborea* and *A. nodosum* Alginates (Initial Propranolol Concentration 0.001M)

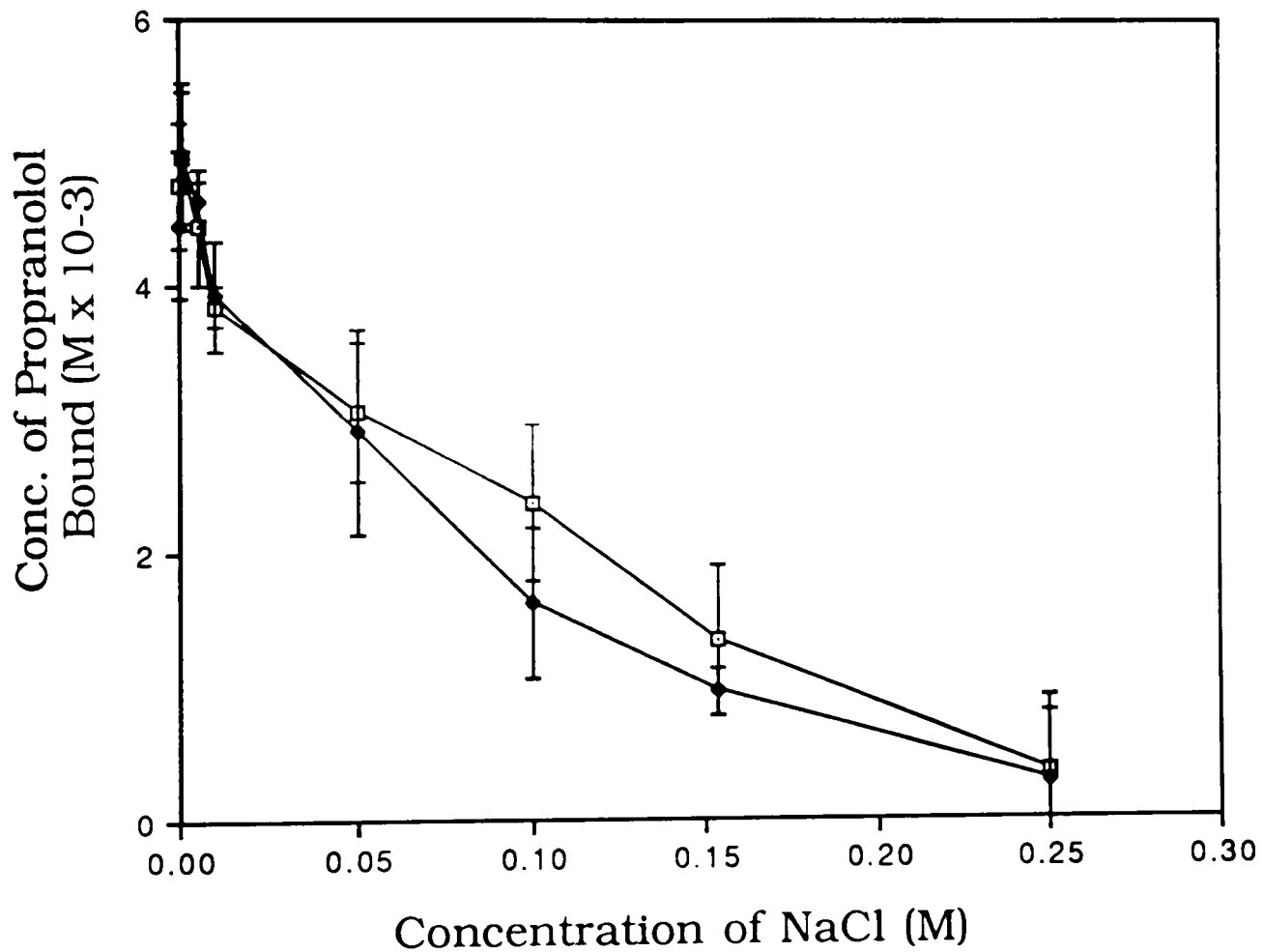


Alginate Conc. = 1gL⁻¹

—□— *L. hyperborea*

—◆— *A. nodosum*

Figure 4.14 The Effect of Added Sodium Chloride on the Binding of Propranolol to *L. hyperborea* and *A. nodosum* alginates (Initial Propranolol Concentration 0.02M)



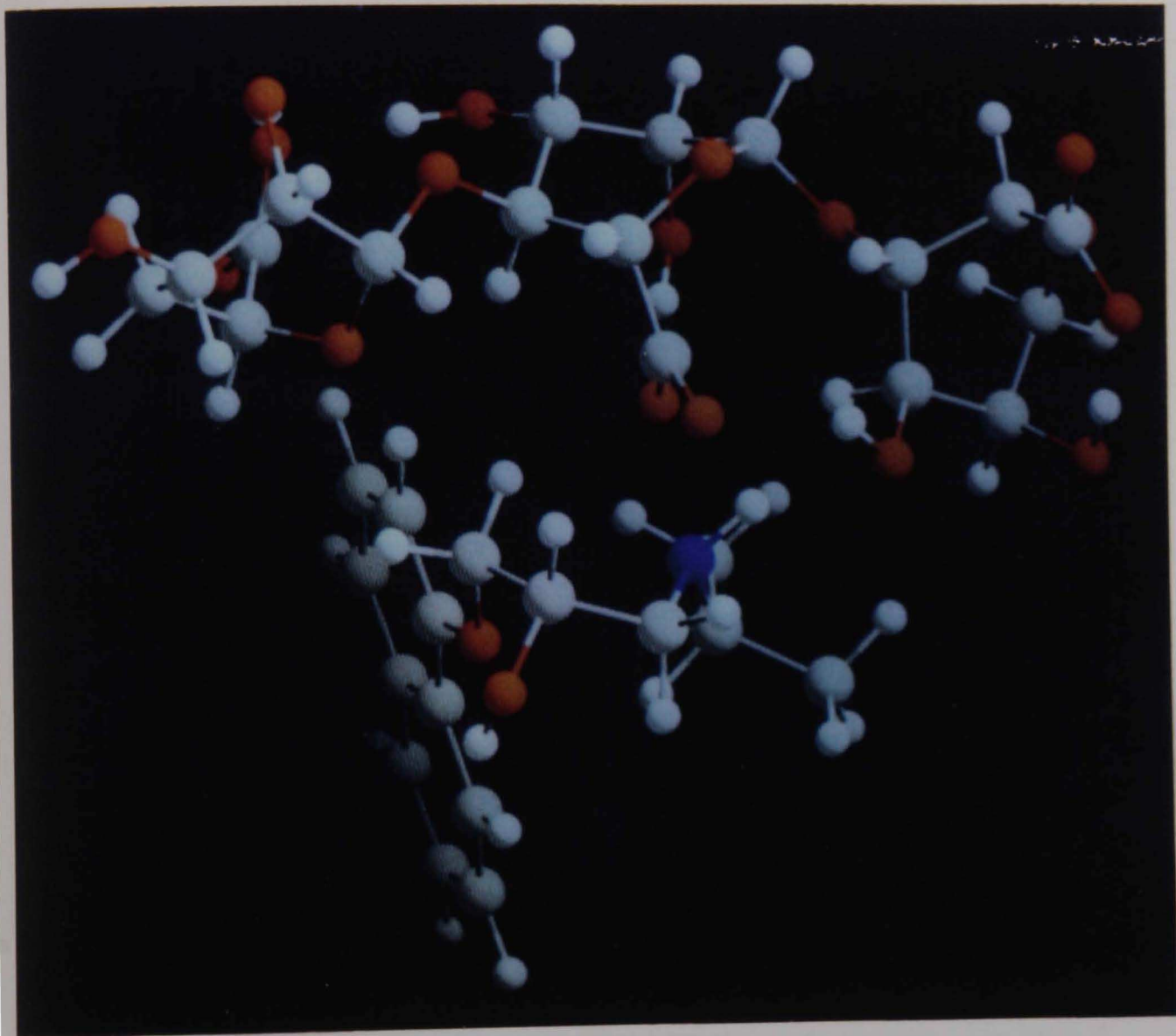
Alginate Conc. = 1gL⁻¹

—□— *L. hyperborea*

—●— *A. nodosum*

Figure 4.15

The Possible *In Vacuo* Three-Dimensional
Structure of a Propranolol/Alginate
Complex as Modelled using the 'COSMIC'
Molecular Modelling System.



4.6 DISCUSSION

The type of binding occurring in a given system can generally be recognised from the saturation curve ('binding isotherm') and may be confirmed by the use of Scatchard or Double Reciprocal plots. As indicated by Equations 4.4 and 4.5, the Scatchard plot and the Double Reciprocal plot would be expected to yield straight lines for hyperbolic binding. However, it sometimes happens that these binding plots do not yield straight lines but instead are curved. This reflects the fact that hyperbolic binding is not occurring and that the ligand sites on the macromolecule are not equivalent.

For example, in the case of oxygen binding to haemoglobin, it is found that the saturation curve is sigmoid in shape. This is because haemoglobin consists of four sub-units held together by non-covalent bonds. Haemoglobin is said to show 'positive co-operativity', that is, binding of the first oxygen molecule enhances the binding of the subsequent ones. The opposite of the 'positive co-operativity' phenomenon is that of 'negative co-operativity', where the binding of the first ligand molecule discourages binding of subsequent ones. This is thought to occur in the binding of NAD^+ to rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Price and Dwek 1979).

Two main theories exist to account for the different types of binding (Price and Dwek 1979). Both stress the importance of changes in shape and conformation of a macromolecule associated with the binding of a ligand. The 'concerted' theory of Monod, Wyman and Changeux is based on the proposition that when a ligand binds to one sub-unit (for example, the hexose residue of an alginate) in a multi-sub-unit macromolecule, all the sub-units change their shape in a 'concerted' fashion. In the 'sequential' model of Koshland, Nementy and Filmer, the conformational changes are thought to be sequential, that is, the ligand changes the conformation of only the sub-unit to which it binds. This conformational change then alters the interaction(s) between the

sub-unit and its neighbouring sub-units making the binding to the latter either stronger or weaker. It should also be remembered that the ligand binding sites on certain macromolecules may be non-equivalent even in the absence of ligands, which could result in non-hyperbolic binding in the presence of a ligand.

Typical saturation curves for hyperbolic binding, positive co-operativity and negative co-operativity are illustrated in Figure 4.16. However, it can be seen from Figure 4.16 that it can be difficult to distinguish between curve 1 (hyperbolic binding) and curve 3 (negative co-operativity). For this reason, a Scatchard plot and/or a Double Reciprocal plot are useful in elucidating the type of binding that may be occurring and also for the determination of 'n', the number of binding sites. Figure 4.17 illustrates a typical Double Reciprocal plot for each of the three forms of binding, whilst Figure 4.18 shows the type of Scatchard plot obtained for each.

From the shape of the binding isotherms for the binding data from the experiments undertaken in this study (Figures 4.7 and 4.8, for the binding of propranolol to *L. hyperborea* and *A. nodosum* alginates respectively), it could be inferred that the type of binding was either hyperbolic or negative co-operativity. If hyperbolic binding was occurring, it would be expected that a Scatchard plot and a Double Reciprocal plot would yield a straight line. However, as can be seen from Figures 4.9 to 4.12, this was not the case.

Thus, comparing the results obtained in Figures 4.9 to 4.12 with the example plots illustrated in Figure 4.16 to 4.18, it would appear that negative co-operativity is occurring when propranolol binds to alginate. This means that the binding of one propranolol molecule to a hexose unit on the alginate makes it more difficult for subsequent propranolol molecules to bind. It is possible to determine 'n', the number of binding sites on the alginate for binding of propranolol. From extrapolation of the curves in Figures 4.9 to 4.12, 'n'=1. This indicates that one propranolol molecule binds with one hexose residue from the alginate. This is in keeping with the chemical structures of propranolol and alginate, since an

Figure 4.16
Saturation Curve

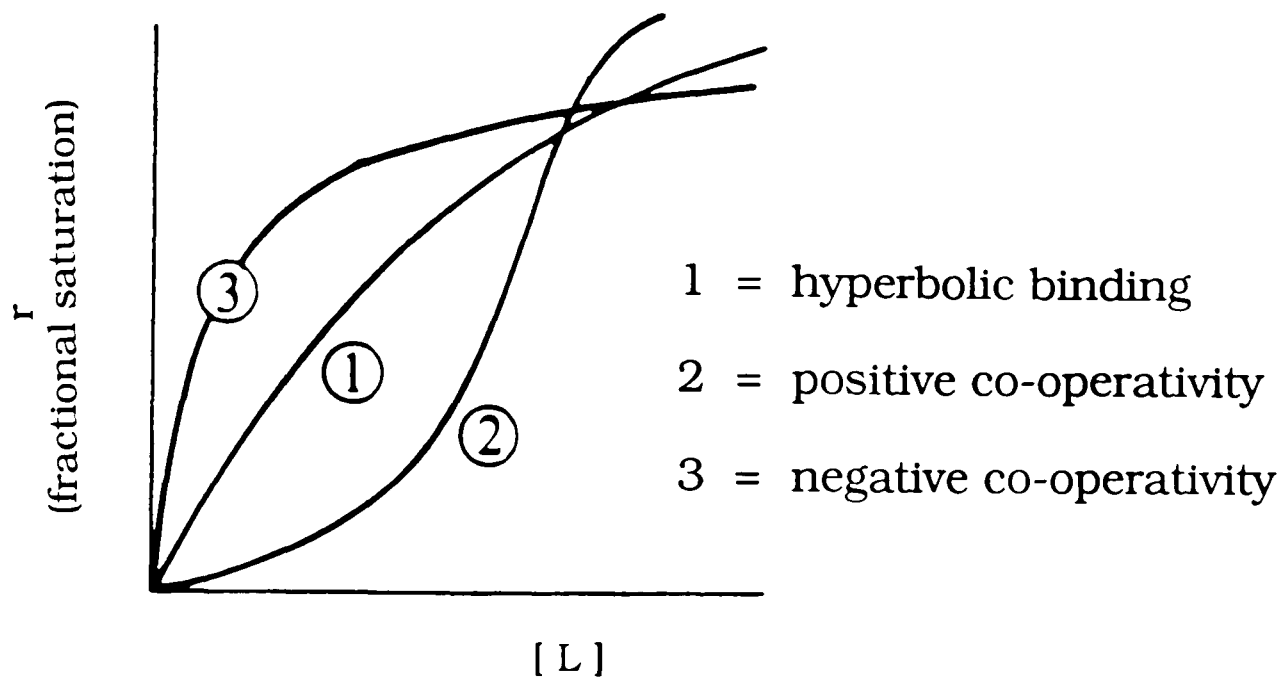


Figure 4.17
Double Reciprocal Plot

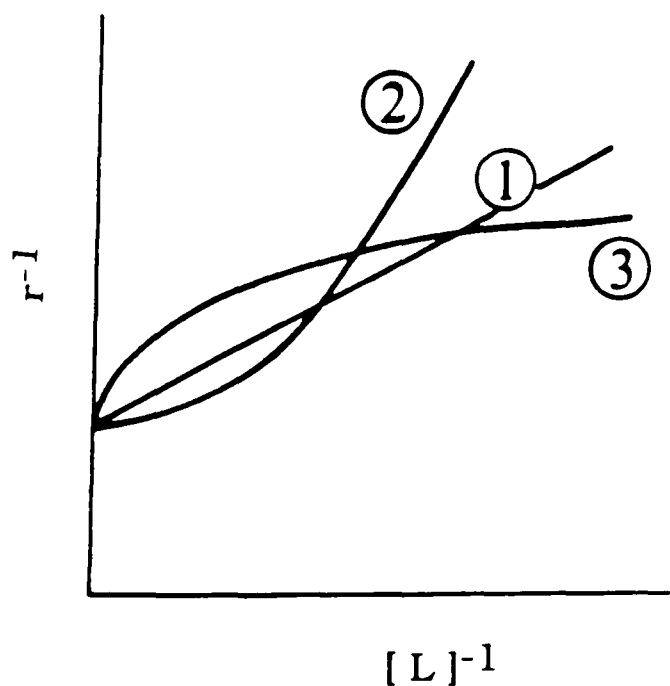
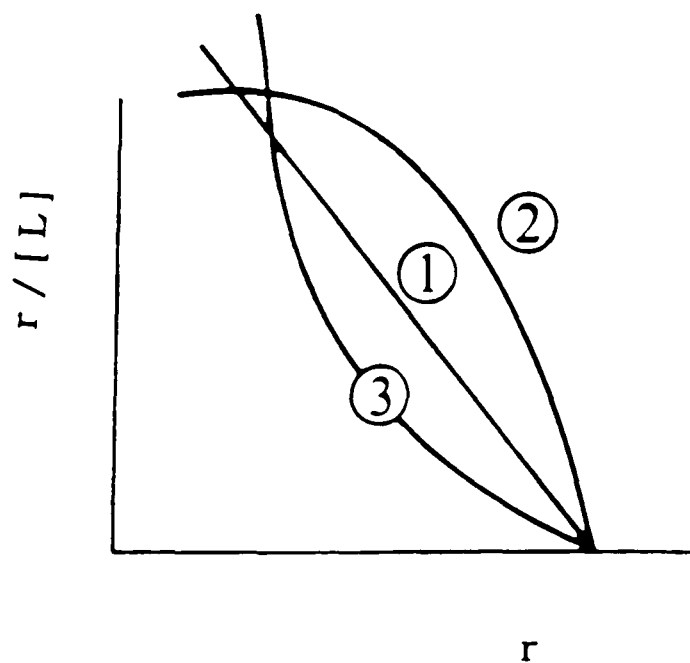


Figure 4.18
Scatchard Plot



r = number of moles of L bound
per mole of macromolecule
 $[L]$ = concentration of free ligand

ionic interaction is possible between the positively-charged tertiary amine group of propranolol (Figure 4.1) and the negatively-charged carboxyl group on each hexose unit of the alginate (Figure 4.2).

Molecular modelling was carried out in order to try to visualise the interaction between alginate and propranolol *in vacuo* and in particular to try to discover why negative co-operativity was occurring. From Figure 4.15, if the carboxyl group on the alginate interacting with the tertiary amine group on the propranolol is modelled, it would appear that the presence of a propranolol molecule interacting with the carboxyl group would make it stereochemically difficult for another propranolol molecule to bind to the adjacent carboxyl group.

However, the molecular model shown in Figure 4.15 does not take into account the aqueous environment in which the binding experiments were carried out, and it is postulated that, in the presence of water, the alginate molecules would be much more extended than those shown, thereby theoretically allowing a propranolol molecule to bind to every carboxyl group on the alginate. Thus, at the saturation point, it is possible that each carboxyl group on the alginate is interacting with the tertiary amine group of one propranolol molecule, in keeping with the 'one-to-one binding' (where 'n' = 1) described above. However, the negative co-operativity observed in the dialysis experiments might be expected, due to steric hindrance from the large naphthalene group on the propranolol molecules making it difficult for further propranolol molecules to bind to adjacent alginate carboxyl groups.

A major point to note is that no difference was observed in the binding of propranolol to the high-G alginate, *L. hyperborea*, (M:G ratio = 35:65) compared to its binding with the high-M alginate, *A. nodosum* (M:G ratio = 61:39). This is in contrast to the interaction of alginate with, for example, calcium ions, where the interaction is much stronger with an alginate rich in guluronic-acid, resulting in the formation of strong alginate gels (Chapter 1). This indicates that propranolol appears to have the same affinity for

binding to G-blocks as to M-blocks or mixed M-G blocks and for this reason, in the *in vitro* and *in vivo* experiments which follow, only the high-G alginate, *Laminaria hyperborea* was studied.

Figures 4.13 and 4.14 demonstrate the effect of added sodium chloride on the binding of propranolol (0.001M and 0.02M respectively) to alginate (1gL^{-1}). As the concentration of sodium chloride is increased, the binding of propranolol to the alginate is gradually decreased, until at a concentration of 0.25M sodium chloride all the propranolol on the alginate has been replaced by Na^+ ions and all the precipitate has disappeared. The fact that the Na^+ ions are able to displace propranolol from an alginate complex provides a possible mechanism for controlled release of propranolol *in vivo*. Many body fluids, for example nasal secretions, contain sodium ions, together with other positive counter-ions such as calcium and magnesium. If the propranolol was administered as an insoluble complex with alginate (for example, as a suspension) it can be seen that any counter-ions present could diffuse in and release propranolol from the alginate, with the possibility of controlled release if the process is sufficiently slow. The following chapters will investigate this possibility for nasal delivery of propranolol.

4.7 CONCLUSIONS

The interaction between propranolol and alginate is thought to be ionic in nature, with the positively-charged tertiary amine group on the propranolol molecules interacting with the negatively-charged carboxyl group on each hexose residue of the alginate molecule. The results from the binding experiments indicated that, at the saturation point, stoichiometric 'one-to-one' binding was taking place, with one propranolol molecule bound to one carboxyl group on each hexose unit of the alginate chain. Analysis of the binding isotherm before saturation indicated that 'negative co-operativity' was occurring, such that the presence of one propranolol molecule binding to a carboxyl group on the alginate

made it more difficult for subsequent propranolol molecules to bind to adjacent carboxyl groups.

No difference in the degree of binding of propranolol to the high-G or high-M alginate was observed, in marked contrast to the interaction of alginate with, for example, calcium ions, where the interaction is much stronger with a high-G alginate.

Molecular modelling was carried out in an attempt to visualise the possible three-dimensional structure of the ionic complex formed between propranolol and alginate. The theoretical model produced supported the experimental finding that negative co-operativity was occurring, since it demonstrated that the binding of the relatively 'bulky' propranolol molecule to the alginate would make it stereochemically difficult for subsequent propranolol molecules to bind to carboxyl groups on adjacent hexose residues.

The interaction and formation of an insoluble propranolol/alginate complex could be reversed by the addition of sodium counter-ions, which displace the propranolol from the alginate and dissolve the complex by the formation of soluble sodium alginate. This should provide a mechanism for controlling the release of propranolol *in vivo* and it is proposed to investigate it further.

CHAPTER 5 PREPARATION AND CHARACTERISATION OF A PROPRANOLOL/ALGINATE COMPLEX

5.1 INTRODUCTION

In order to prepare a dosage form which could be conveniently administered to rats, it was decided to prepare a dried alginate/propranolol complex which could then be reconstituted. Several techniques for achieving this were investigated, including filtration and oven drying, but these resulted in either loss of the precipitate during preparation, or the formation of a charred brown complex.

The technique eventually chosen for preparation of the dosage form was freeze-drying. The following chapter describes the formation of a freeze-dried propranolol/alginate complex which was characterised in terms of particle-size, propranolol content and drug release *in vitro* in the presence of sodium chloride ions.

5.2 FORMATION OF THE COMPLEX

5.2.1 Introduction

The ratio of propranolol to alginate used in preparing the precipitate was determined using the results obtained from the equilibrium dialysis experiments described in Chapter 4. A point on the plateau of the binding isotherm plot for *Laminaria hyperborea* alginate (Figure 4.2) where 100% of the alginate hexose residues were occupied by propranolol ions ('one-to-one' binding) was chosen. The concentration of alginate at this point was 1gL^{-1} and the concentration of propranolol was 0.02M.

5.2.2 Materials

Propranolol hydrochloride BN 8905237 (CP Pharmaceuticals, Loughborough);

Laminaria hyperborea alginate BN 902-282-04 (Protan Laboratories, Norway);

AnaLar water BN 3137510L (BDH, Poole, Dorset).

All were used as received.

5.2.3 Methods

A propranolol/alginate complex was prepared as follows:

Alginate solution (500mL; 2gL⁻¹) was mixed with propranolol solution (0.04M) to produce a cloudy mixture, which was stirred for 45 minutes at 25°C, and then left to allow the precipitate to settle. The supernatant was carefully poured off, and the slurry-like precipitate centrifuged at 3000 r.p.m. for 5 minutes. Further supernatant was poured off and analysed in a UV spectrophotometer at 288nm for propranolol content. The precipitate was then washed with deionised water to remove any free propranolol and soluble propranolol/alginate complex, then re-centrifuged. This washing and centrifugation procedure was repeated five times until the UV analysis of the supernatant indicated that the concentration of propranolol in the supernatant was negligible.

After the final washing, the slurry was centrifuged at 3000 r.p.m. for 30 minutes and excess water removed with a pipette. The complex was then transferred to a 100mL round-bottomed flask and frozen *in situ* in liquid nitrogen. It was then freeze-dried at -60°C for 24 hours.

5.2.4 Results

A white 'spongy' freeze-dried complex of alginate and propranolol was produced, which was characterised as described in section 5.3.

5.3 CHARACTERISATION OF THE PROPRANOLOL/ALGINATE COMPLEX

The freeze-dried complex was characterised in terms of:

- (i) particle size in suspension (section 5.3.1);
- (ii) propranolol content (section 5.3.2);
- (iii) *in vitro* drug release (section 5.3.3).

5.3.1 Particle Size in Suspension

5.3.1.1 Introduction

Measurements of the particle size of drugs are made routinely in the pharmaceutical industry. Particle size can have an important effect on the dissolution rate of a drug, since any reduction in particle size has the effect of exposing increasing amounts of surface of the drug to the solvent. *In vivo*, this may determine the rate of absorption of a drug, the speed of onset of effect of the drug, the duration of therapeutic response and the occurrence of toxic side-effects (Florence and Attwood 1985).

For a spherical particle, it is possible to describe particle size in terms of diameter, but this becomes ambiguous if the particle is irregular in shape. For this reason, the particle size of a non-spherical particle is quoted as the diameter of a sphere which is in some way equivalent to that particle. Such a sphere is termed an 'equivalent sphere' and the diameter is an 'equivalent diameter'. In these experiments, the particle size is expressed as the 'volume equivalent diameter' (d_v). This means that if an irregular particle is quoted as having a volume equivalent diameter of, say, 1 micrometre, it has the same volume as a spherical particle of diameter 1 micrometre (Washington 1992).

5.3.1.2 Materials and Equipment

Propranolol/alginate complex (section 5.2 above);
Sodium chloride BN 6794910K (BDH, Poole, Dorset);
AnaLar water BN 3137510L (BDH, Poole, Dorset);
Malvern Mastersizer particle sizer, Malvern Instruments, Malvern,
England.

All reagents were used as received.

5.3.1.3 Methods

The freeze-dried propranolol/alginate complex was prepared as in section 5.2. This was re-suspended in AnaLar deionised water by shaking for five minutes by hand and the particle size range of the suspension was determined using a Malvern 'Mastersizer'.

The reader is referred to Washington (1992) for a detailed description of the Malvern Mastersizer and its use in determining particle size. In summary, the technique involves passing a beam of light from a laser source through the sample which is stirred continuously to keep the particles in suspension. Light scattered by the sample passes onto a detector and the use of computer software enables the calculation of a particle size distribution from this scattering pattern.

5.3.1.4 Results

Figures 5.1 and 5.2 show the particle size distribution and the cumulative particle size distribution respectively for the freeze-dried propranolol/alginate complex re-suspended in AnaLar deionised water.

Using the computer attached to the Mastersizer, it was calculated that the average size of the propranolol/alginate complex particles in suspension was 22.5 μm (volume equivalent diameter). 80% of particles in the sample were within the size range 4.6 μm to 82 μm ; 10% of particles were larger than 82.0 μm and 10% of particles were smaller than 4.6 μm .

Figure 5.1 Particle Size Distribution of Freeze-Dried Propranolol/Alginate Complex Suspended in Deionised Water

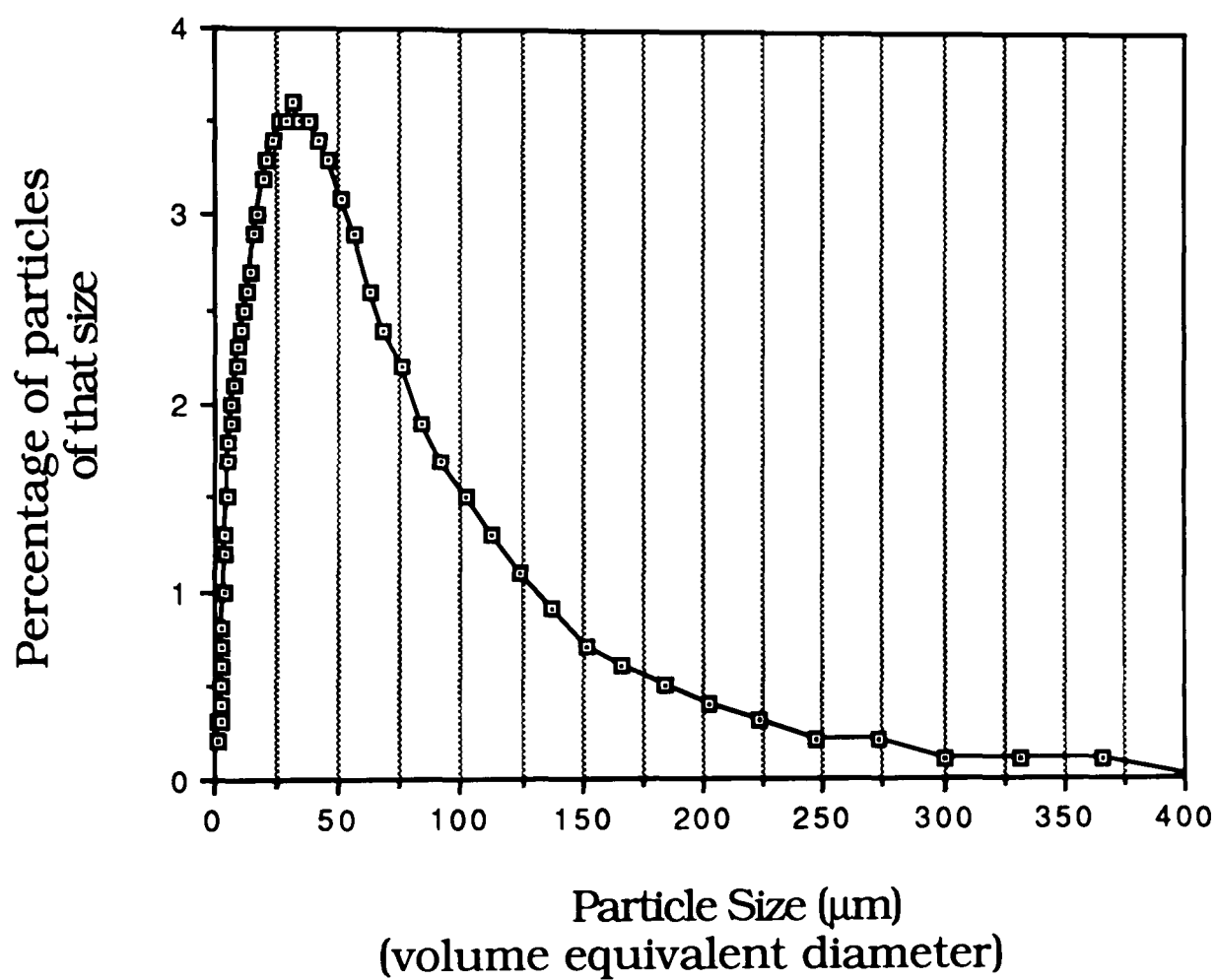
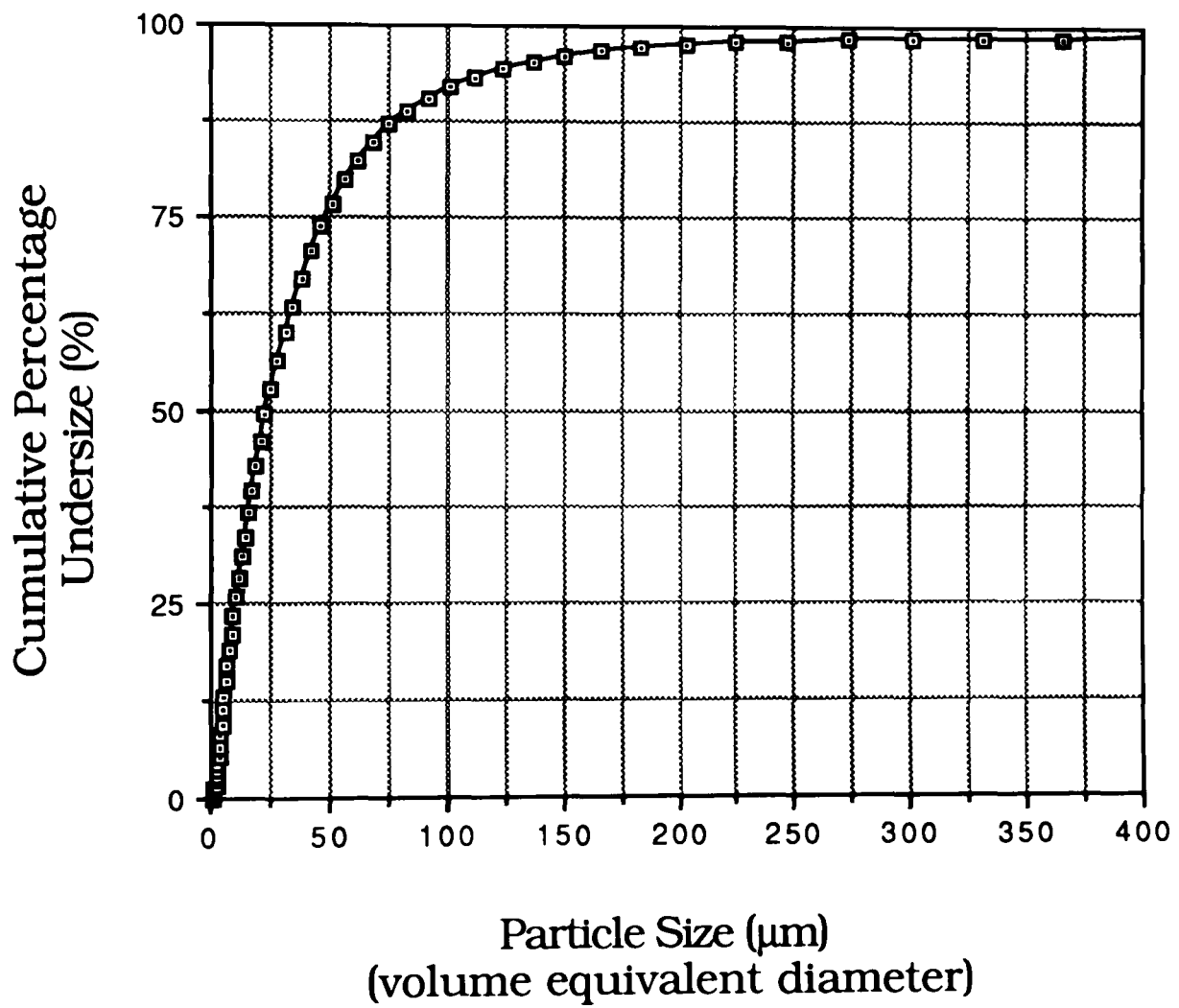


Figure 5.2 Cumulative Particle Size Distribution of Freeze-Dried Propranolol/Alginate Complex Suspended in Deionised Water



5.3.2 Propranolol Content

5.3.2.1 Materials and Equipment

Propranolol/alginate complex (section 5.2 above);

Sodium chloride BN 6794910K (BDH, Poole, Dorset);

AnaLar water BN 3137510L (BDH, Poole, Dorset);

Uvikon 860 UV Spectrophotometer, Kontron Instruments, England.

All reagents were used as received.

5.3.2.2 Methods

In order to determine the propranolol content of the complex, a calibration curve for propranolol was constructed (Figure 5.3). Three different known weights of the complex (20mg, 33mg and 40mg) were then dissolved in 1000mL of 0.9% sodium chloride solution and analysed in the UV spectrophotometer at 288nm for propranolol concentration. Each determination was performed in triplicate.

5.3.2.3 Results

The results from the determination of propranolol content are shown in Table 5.1.

Figure 5.3 Calibration Curve for UV Spectrophotometric Determination of Propranolol at 288nm

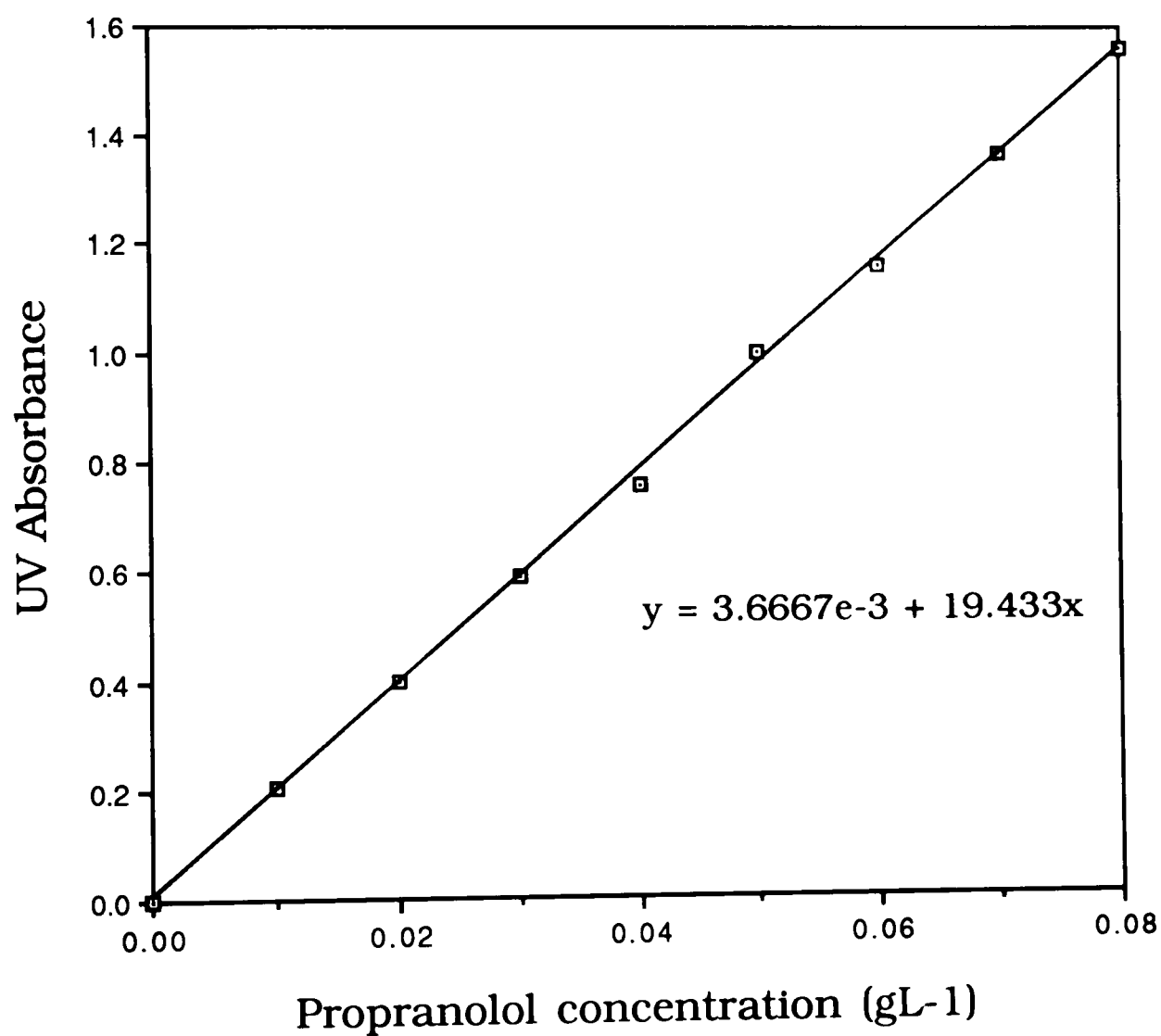


Table 5.1 Determination of the Propranolol Content of the Propranolol/Alginate Complex

<u>Weight of precipitate dissolved in 1000mL (g)</u>	<u>UV Abs. at 288nm (n=3, SD≤0.005)</u>	<u>Concentration of propranolol (from calibration curve) (gL⁻¹)</u>	<u>Proportion of propranolol in precipitate. (%w/w)</u>
0.020	0.259	0.0131	65.5
0.033	0.428	0.0218	66.1
0.040	0.516	0.0263	65.8
			—
			Mean = <u>65.8%w/w</u>

The mean percentage of propranolol in the precipitate was measured as 65.8%w/w. This is slightly higher than the theoretical calculation of propranolol content based on molecular weights, assuming that one molecule of propranolol is bound to one carboxyl group on each hexose unit on the alginate. In this case, the theoretical proportion of propranolol in the complex would be calculated as 60%w/w as follows:

MW propranolol = 296;

MW of one guluronic or mannuronic acid residue = 200;

Therefore, assuming one-to-one binding, the percentage of propranolol within the complex would be:

$$296 / (296 + 200) \times 100 = \underline{60\%w/w}.$$

However, it is probable that, despite repeated washing of the precipitate during its preparation to remove free propranolol, some free propranolol remained which was freeze-dried with the propranolol/alginate complex.

5.3.3 IN VITRO DRUG RELEASE STUDIES FROM THE PROPRANOLOL/ALGINATE COMPLEX

5.3.3.1 Introduction

In vitro experiments were set up to investigate the rate of release of propranolol from the propranolol/alginate complex across a semi-permeable membrane. The system employed was dialysis across a semi-permeable Visking membrane (of the same type used for the equilibrium dialysis experiments in Chapter 4) into 0.9% sodium chloride solution.

5.3.3.2 Materials

Propranolol/alginate complex formed in section 5.2 above;
AnaLar water BN 3137510L (BDH, Poole, Dorset);
Sodium chloride (BDH, Poole, Dorset);
Visking dialysis tubing (cut-off point 5,000 Daltons).

5.3.3.3 Methods

The following formulations were tested:

- (i) Propranolol solution 1gL^{-1} ;
- (ii) Propranolol solution 5gL^{-1} ;
- (iii) Propranolol solution 10gL^{-1} ;
- (iv) Alginate/propranolol complex formulated as a suspension in deionised water (equivalent to 10g of propranolol in 1L of suspension);
- (v) Alginate/propranolol complex formulated as a suspension in isotonic glycerol solution (equivalent to 10g of propranolol in 1L of suspension).

For each of the above formulations, 5mL of solution/suspension was placed in a dialysis bag (100mm long x 50mm

diameter of the same Visking tubing used for the equilibrium dialysis experiments described in Chapter 4). This was immediately placed in a flask containing 500mL of 0.9%w/v sodium chloride solution, previously heated to 37°C and placed in a shaking water bath at 37°C.

3mL samples were withdrawn from the flask at intervals up to 150 minutes and analysed for propranolol concentration at wavelength 288nm. 3mL of 0.9%w/v sodium chloride solution was added to the flask after each sampling in order to keep the volume of fluid constant. Each experiment was performed in triplicate. Standard deviations were less than 0.5% in all cases.

5.3.3.4 Results

Figure 5.4 shows plots of percentage of drug released versus time for the solutions containing 1gL^{-1} , 5gL^{-1} and 10gL^{-1} of propranolol. Since the percentage release of propranolol over time was similar for all these solutions and appeared to be dependent only on the rate of diffusion of the drug across the membrane, it was decided to compare only the 10gL^{-1} solution with suspensions of the propranolol/alginate complex containing an equivalent amount of propranolol.

Figure 5.5 shows the *in vitro* release profile for the propranolol/alginate complex 15.2gL^{-1} in deionised water (equivalent to 10g propranolol in 1L of suspension) compared with that for propranolol solution 10gL^{-1} . Figure 5.6 shows the *in vitro* release profile for the propranolol/alginate complex 15.2gL^{-1} in isotonic glycerol solution (equivalent to 10g propranolol in 1L of suspension), compared to the profile for propranolol solution 10gL^{-1} .

Figure 5.7 is a comparison of the release profile of propranolol from the complex formulated in AnaLar deionised water and in isotonic glycerol solution.

Figure 5.4 Release Profile of Propranolol from Solution

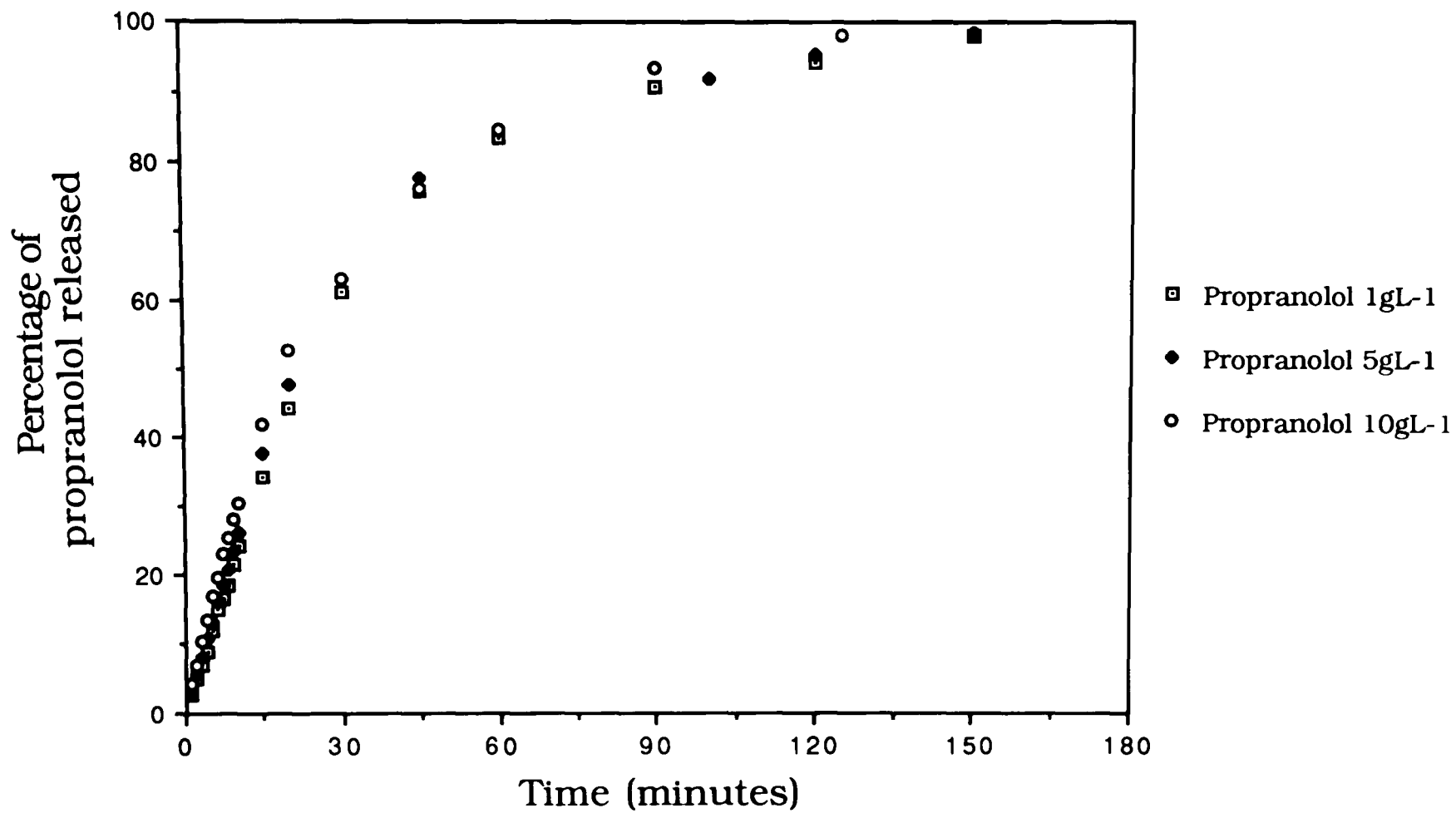


Figure 5.5 Release Profile of Propranolol from Propranolol/Alginate Complex (Suspension in Deionised Water)

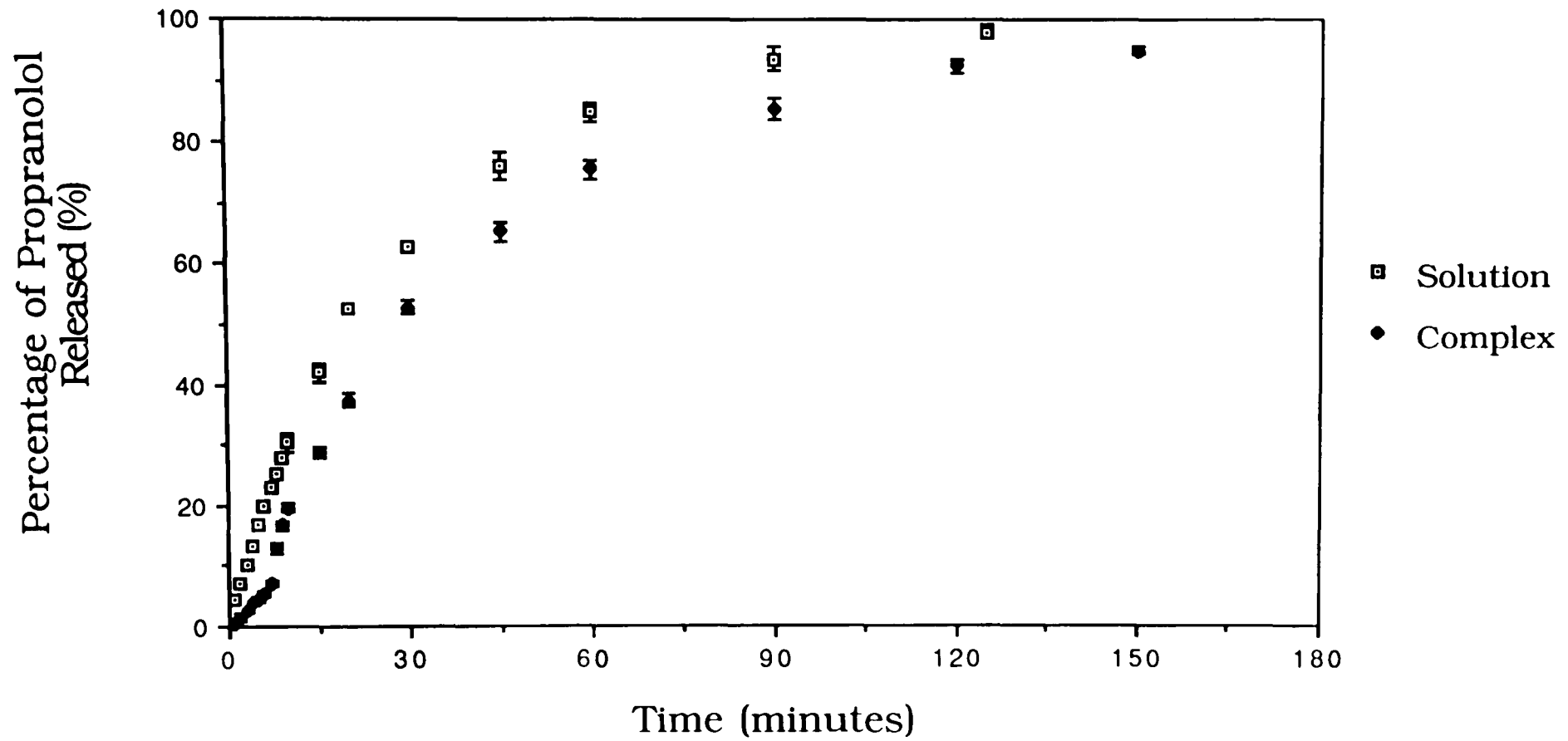


Figure 5.6 Release Profile of Propranolol
from Propranolol/Alginate Complex
(Suspension in Isotonic Glycerol)

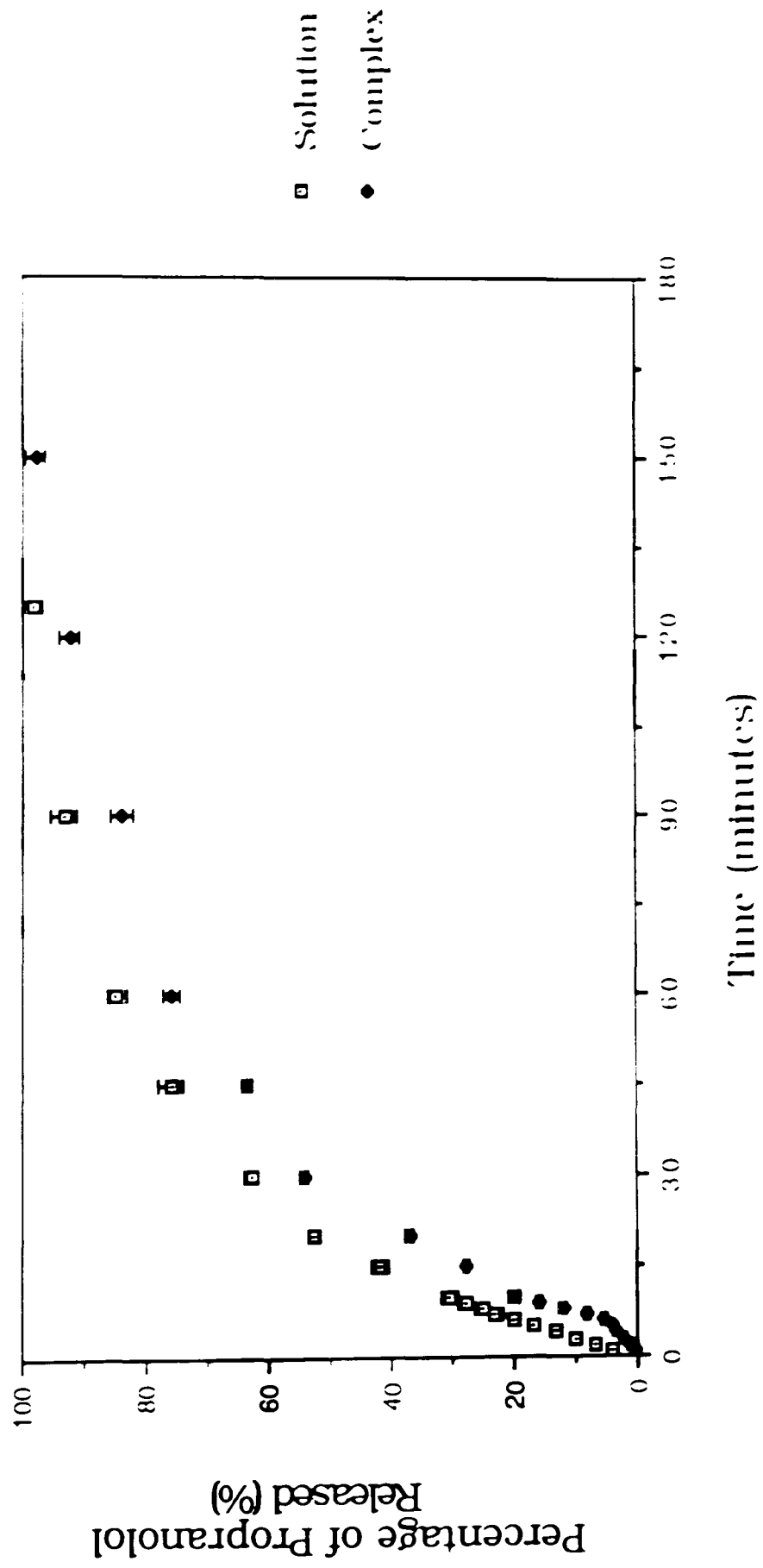
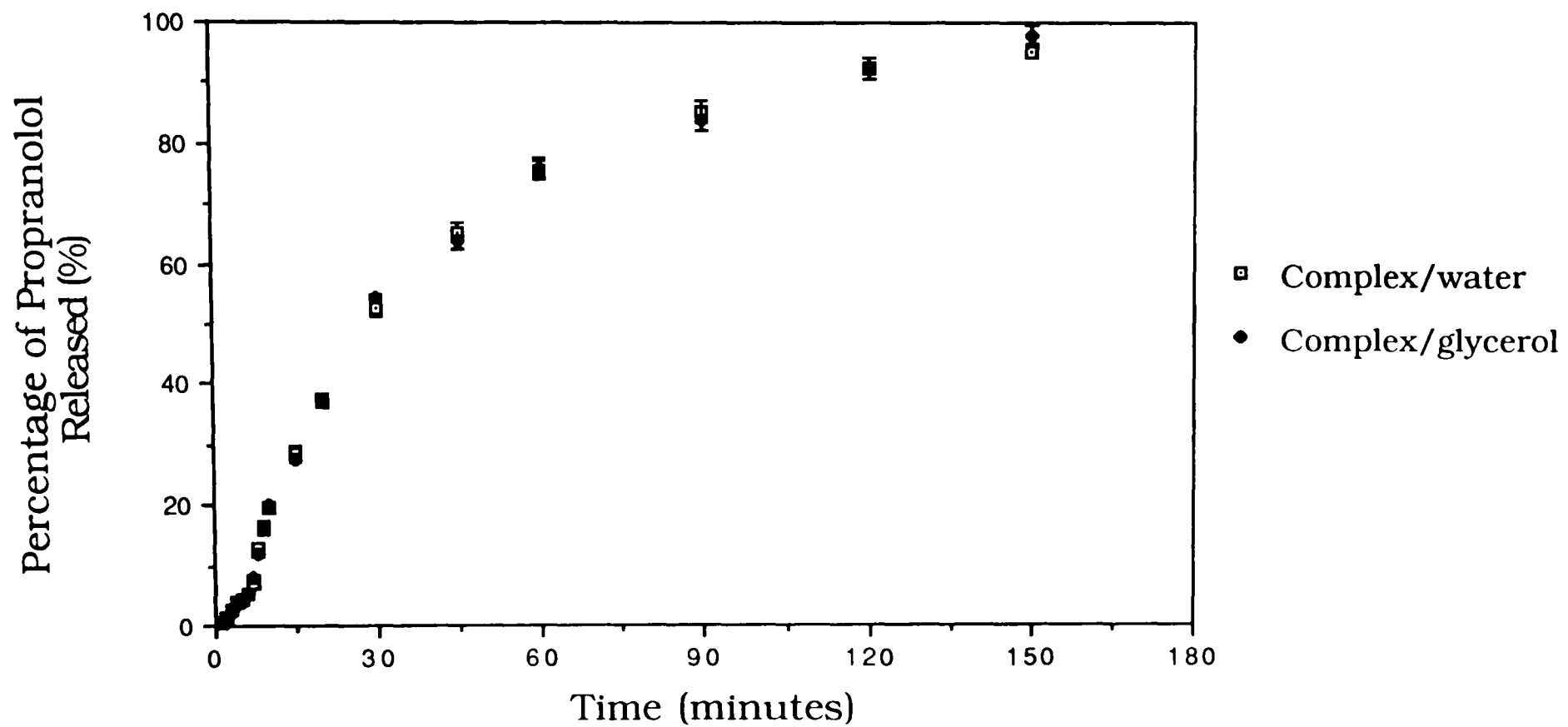


Figure 5.7 Comparison of Release Profile for Propranolol/Alginate Complex in Deionised Water and in Isotonic Glycerol



5.4 DISCUSSION

From Figures 5.5 and 5.6 it can be seen that there is a lag of approximately 5-6 minutes in the release profile for the propranolol/alginate complex compared with the propranolol solution. The profile for the solution would be expected to be dependent only on the rate of diffusion of propranolol through the semi-permeable dialysis membrane, but for the propranolol/alginate complex, several processes may be occurring. Na⁺ ions diffuse into the dialysis bag and since the affinity of the sodium ions for alginate is greater than that of the propranolol ions (Chapters 3 and 4), ion exchange occurs releasing drug and dissolving the propranolol/alginate complex. The propranolol ions can then diffuse out of the dialysis bag into the sodium chloride solution. The lag time of 5-6 minutes observed suggests that this time is taken whilst the precipitate is ion-exchanging and dissolving, as subsequently the profile is almost identical to that of the solution.

Therefore, it would appear that when propranolol is formulated as a complex with alginate in suspension, there is some evidence for delayed release relative to that of a propranolol solution. Furthermore, from Figure 5.7 it can be seen that the presence of glycerol as an agent to adjust tonicity did not appear to affect the release of propranolol from the complex compared to the release of propranolol from the complex formulated in deionised water.

5.5 CONCLUSIONS

A freeze-dried propranolol/alginate complex has been prepared from propranolol hydrochloride and the sodium alginate *Laminaria hyperborea*.

When tested *in vitro* for release of propranolol across a semi-permeable membrane, it was found that release of propranolol

from the complex (formulated as a suspension in either AnaLar deionised water or in isotonic glycerol) was slightly delayed compared with release from a propranolol solution.

The release of propranolol from the formulations was then assessed *in vivo* using the anaesthetised rat as an animal model for nasal delivery. This is described in the following chapter.

CHAPTER 6

IN VIVO DRUG ABSORPTION STUDIES

6.1. INTRODUCTION

Nasal delivery in the rat was chosen as a model *in vivo* system in which to investigate the potential of alginate ionic complexes to control drug absorption. Propranolol is a relatively small (MW 295), lipophilic drug ($\log P$ octanol/water = 3.36) with a pKa of 9.45. It is rapidly absorbed intranasally in rats and its systemic bioavailability by this route is nearly 100% relative to an intravenous dose (Hussain et al 1980b).

6.1.1 Nasal delivery - background

As a site for the absorption of various compounds, the nasal cavity is not a recent discovery. For example, the South American Indians and certain African Witchdoctors are reported to have used snuffs made from medicinal plants in the treatment of disease, and the use of tobacco snuff in England dates back to the seventeenth century, when it was introduced by Sir Walter Raleigh (Bowman and Rand 1980). More recently the abuse of cocaine and similar drugs by 'snorting' has become more widespread.

It was not until the twentieth century that the first cases of compounds being systemically absorbed after intranasal (IN) administration were reported in scientific literature. For example, in 1922, Blumgart demonstrated the antidiuretic effect of intranasal pituitary extract employed in the treatment of diabetes insipidus, and Cohen et al (1930) reported the absorption of ragweed pollen introduced into the nasal cavity. In 1932, several workers delivered insulin intranasally to human diabetics, by combining it with

saponin, although this resulted in symptoms of congestion and rhinitis.

Over the last decade, the number of publications concerning nasal absorption has increased rapidly. Two comprehensive reviews of substances applied to the nose for both topical and systemic effects have been published (Chien 1985; Chien and Chang 1987). These authors have outlined the vast range of compounds which have been administered via the nose with varying degrees of success, for example, cardiovascular drugs, antimicrobial biologicals, CNS stimulants and numerous hormones, to name but a few. Despite all the scientific publications, there still remains only a scant number of drugs which are administered nasally for their systemic effects. These include vasopressin (anti-diuretic hormone) and desmopressin which are both used in the treatment of pituitary diabetes insipidus, and oxytocin which is used to induce labour in pregnant women.

Traditionally, the intranasal route has been associated with the topical treatment of local conditions, for example allergic rhinitis, although it has been shown to be suitable for certain drugs intended to have a systemic effect, but which demonstrate poor oral bioavailability. Therefore, as an alternative to the injection of drugs, the nasal route is becoming increasingly popular. This is because, in common with other parenteral routes, drugs crossing the nasal mucosa pass directly into the systemic circulation (Parr 1983). For many drugs, the rate and extent of IN absorption, closely resemble that of an intravenous (IV) dose, but for the patient, the nasal route offers a better alternative to repeated injection. In addition, the nose is easily accessible for drugs to be administered in the form of drops, sprays or gels, and as a surface for absorption the nasal cavity is well supplied with blood and has numerous cells with microvilli or cilia. Compared with the gastrointestinal tract, there is a much more favourable environment, since breakdown of drug in the gut lumen or wall, and first-pass metabolism by the liver are avoided.

The systemic bioavailability and therefore the efficacy of drugs is often influenced by the route of administration selected. For a

drug to be at its maximum therapeutic efficiency, it should be absorbed rapidly, and administered easily, so that, for many drugs, the nasal route of administration would seem to be an excellent choice. However, a variety of both biological, physico-chemical and pharmaceutical factors can affect the absorption of drugs via the nose into the systemic circulation. These are discussed below for nasal delivery of drugs to humans.

6.1.2 Factors affecting nasal delivery.

6.1.2.1 Structure and function of the nasal cavity

Various texts are available which provide detailed descriptions of the anatomy of the nasal cavity in man (Mygind 1985; Chien and Chang 1987) and in animals (Popp and Martin 1986; Keleman 1947), and the reader is referred to these for more detailed accounts. In summary, the nasal cavity is divided by a midline septum composed of bone and cartilage, with each half being separated into three regions: the vestibule (the region just inside the nose); the olfactory region (covering the roof of the nasal cavity, and some of the upper portions of the nasal septum and lateral walls) and the respiratory region (the rest of the cavity). The absorptive surface is the nasal mucosa, which is composed mainly of epithelial cells. The 'mucociliary apparatus' which is comprised of ciliated epithelial cells and glandular tissue, secretes a layer of mucus onto the surface of the epithelium, which prevents it drying out and, together with coarse hairs in the vestibular region, also forms a defence system for removing inhaled pollutants.

The main function of the nasal cavity is a protective one, whereby inhaled air is filtered, warmed and humidified in preparation for gaseous exchange in the lungs (Mygind 1985). Hairs and mucus within the cavity trap particles, which are then removed either mechanically or by the action of the mucociliary apparatus.

The olfactory function of the nose also acts as a protective mechanism by aiding in the detection of possible poisonous gases (Negus 1958).

It can be seen that the structure of the nasal cavity can represent a barrier to the absorption of drugs, since the drug must pass through both the surface mucus layer and the mucous membrane itself, whilst avoiding mucociliary clearance, before it reaches the systemic circulation.

6.1.2.2. Metabolism within the nasal cavity

Although the total metabolic capacity of the nasal cavity has yet to be defined, a number of enzyme systems have been identified which are thought to have an important function in metabolically inactivating and/or detoxifying foreign substances. Therefore it is possible that various metabolic processes may interfere with the absorption of drugs administered via the nose and may also produce inactive or toxic metabolites.

6.1.2.3. Biological factors

Respiratory infection such as the common cold usually result in the production of either a watery mucus which could cause 'washing out' of the dose, or a thick viscous mucus which could prevent the dose reaching the absorptive surface. In addition, mechanical clearing of the nasal cavity, for example by sneezing or blowing the nose, is a potential source of drug loss.

Certain conditions such as allergic rhinitis have been shown to increase absorption of drugs by an increase in the 'leakiness' of intracellular junctions (Inagaki et al 1985), whilst the presence of polyps or tumours would reduce the volume of the nasal cavity and could interfere with distribution of the dose within the nose.

The blood supply to the nose can be affected by both emotional factors such as stress, and environmental factors, such as

temperature. Exposure to adverse conditions, for example cigarette smoking, over prolonged periods can also result in damage to the nasal mucosa (Proctor 1977).

6.1.2.4. Physico-chemical factors

The passage of a drug from the nasal cavity into the systemic circulation requires it to move from the aqueous boundary layer covering the surface of the nasal mucosa across the lipid barrier membrane, and then to diffuse across the cell and on into the circulation. For this reason, the physicochemical properties of the drug such as its pKa, lipophilicity and molecular size will influence the extent and rate of absorption.

In general, unionised molecules are more readily absorbed across mucosal membranes, so that changes in the pH of either nasal secretions or of the drug solution could affect absorption. The pH of the nasal cavity has been reported to be around 5.5-6.2 in humans and between 7.2-7.4 in the rat (Hirai et al 1981). The absorption of lipophilic drugs will be limited by the rate of diffusion across the aqueous boundary layer, whilst for hydrophilic drugs, partitioning into the lipid membrane will be the rate-limiting step. Although it has been demonstrated that drugs with a wide range of molecular weights have crossed the nasal mucosa, it is generally found that drugs with a molecular weight of greater than 1000 are less well absorbed (McMartin et al 1987).

6.1.2.5. Pharmaceutical factors

The dosage form used to administer a drug to the nose could affect the eventual absorption of the drug from the nasal cavity. For example, it would seem likely that drug molecules would reach the absorptive surface more quickly from aqueous solutions than when having to diffuse through a viscous gel network.

In humans, it is usual to administer nasally-delivered drugs in

an isotonic formulation in order to minimise irritation to the nasal mucosa. However, some workers have demonstrated that absorption of drugs can be modified by using non-isotonic formulations (Daugherty et al 1988).

Finally, the use of excipients (such as surfactants, bile salts, chelating agents, fatty acids or enzyme inhibitors) to enhance nasal absorption is well documented (for example, Lee et al 1991), although certain excipients may be added, for example, to increase the stability or tonicity of a formulation. Therefore, it should be appreciated that added excipients have the potential to affect the absorption of a drug from the nasal cavity.

6.1.3 Nasal delivery of propranolol

Propranolol hydrochloride is usually given as an oral dose, but may also be administered intravenously for the treatment of arrhythmias.

Shand et al (1970) reported that oral administration of propranolol to five human subjects resulted in great variability in the plasma levels measured. Peak plasma levels after an 80mg oral dose varied as much as sevenfold, compared to only a twofold variation in the same subjects after IV administration of a 10mg dose. In the same study it was estimated that the systemic bioavailability of an oral dose of propranolol ranged from 16% to 60% of the bioavailability of the IV dose. The authors suggest that these differences could be the result of extensive first-pass metabolism of propranolol by the liver after an oral dose.

Intranasal (IN) administration of propranolol was investigated as a method of enhancing drug bioavailability and minimising the variation in blood levels seen with other non-parenteral routes (Hussain et al 1979). Propranolol was administered to the nasal cavity of Sprague-Dawley rats with a micropipette. The results indicated that the blood drug levels for IV and IN administration of 1mg doses were identical, whereas oral administration resulted in considerably lower blood levels. The authors concluded that:

(a) Propranolol is rapidly absorbed from the nasal mucosa with peak plasma levels being obtained within five minutes of instillation;

(b) The nasal route for administration of propranolol appears to be as effective as the IV route and superior to the oral route.

In another study by the same workers, the benefits of nasal administration of propranolol were demonstrated further in rats and dogs (Hussain et al 1980a). A sustained-release formulation of the drug incorporated with methylcellulose was administered intranasally. The investigations suggested that the sustained-release preparation resulted in low initial but prolonged blood levels, such that the bioavailability was identical to that of the IV route.

Hussain et al (1980b) also evaluated the nasal absorption of propranolol in human subjects, and compared it with IV and oral administration. Their results demonstrated that IN administration of propranolol hydrochloride in 2% methylcellulose gel produced a blood level identical to that obtained after IV infusion. However, the oral bioavailability from propranolol tablets was found to be only 25%. These findings were in agreement with the previous studies in rats and dogs.

6.1.4 Use of an anaesthetised rat model for intranasal studies

The use of animal models to study the absorption of drugs across the nasal mucosa is well known (Gizurason 1990), with the rat and the dog being the two most commonly used species. For certain nasally administered drugs, animal models allow prediction of *in vivo* profiles in man. For example, Hussain et al (1979, 1980a and 1980b) demonstrated a good correlation between the nasal absorption of propranolol in rats, dogs and man. However, it should be noted that for other drugs, for example insulin, significant differences in absorption between species have been documented (Verhoef et al 1989).

In the work described in this chapter, an *in vivo* anaesthetised rat model modified from that described by Hirai et al

(1981) was used. Possible disadvantages of this model are that various factors including anaesthesia, surgical intervention and orientation of the animal may result in changes in the absorption of drugs through the nasal mucosa. For example, Daugherty et al (1988) reported that absorption of methionyl recombinant human growth hormone was greater from an *in vivo* anaesthetised rat model than from the conscious animals.

6.2 MATERIALS

Sodium hydroxide BN 21694101 (Fisons, Loughborough, Leics.);

Heptane BN 2377520L (BDH, Poole, Dorset);

Iso-amyl alcohol BN 4040032 (Sigma Chemical Co., St. Louis, U.S.A.);

Hydrochloric acid (BDH, Poole, Dorset);

Propranolol hydrochloride BN 8905237 (CP Pharmaceuticals, Loughborough, Leics.);

Bench Centrifuge Centaur 2 (MSE, Loughborough, Leics.);

Perkin-Elmer 3000 Fluorescence spectrometer (Perkin-Elmer, Beaconsfield, Bucks.);

AnaLar deionised water BN 3137510L (BDH, Poole, Dorset).

All materials were used as received.

6.3 METHODS

6.3.1 Study design

The study was designed to compare the intranasal absorption of propranolol from a solution with its absorption from a complex with alginate. Many routes of drug administration, for example injections, require formulations which are isotonic with blood serum, in order to avoid haemolysis (if the solution is hypotonic) or damage to vein walls (if the solution is hypertonic). Nose drops are usually formulated to be isotonic with natural nasal secretions. Whilst this is not essential, it reduces the transient stinging and soreness

associated with administration of non-isotonic solutions to humans. It was therefore decided to prepare an isotonic formulation of the propranolol/alginate complex using glycerol solution, in order to investigate if an osmotic effect would influence drug absorption from the complex. Glycerol was chosen since it is non-ionic and it had previously been demonstrated (section 3.2.5) that ionic salts such as sodium chloride (which are often used to adjust tonicity) caused the propranolol/alginate complex to dissociate. The concentration of glycerol solution used was 26gL^{-1} (that is, the concentration stated as being iso-osmotic with blood by the Pharmaceutical Handbook Nineteenth Edition (1980)).

6.3.2 Preparation of Dosing Solutions/Suspensions

The alginate/propranolol complex was prepared as described in section 5.2. Three different formulations were administered:

- (i) Dose group 1: propranolol solution in deionised water;
- (ii) Dose group 2 : propranolol suspension (containing propranolol/alginate complex in deionised water;
- (iii) Dose group 3 : propranolol suspension (containing propranolol/alginate complex in isotonic glycerol solution).

6.3.3 Animal Model - Surgical Procedures

Anaesthetised male Sprague-Dawley rats (body weight 250g to 330g) were used in all experiments. The rats were starved for 12 hours prior to dosing. Anaesthesia was induced by intraperitoneal administration of urethane (1.25g/kg of either a 10%w/v or 40%w/v solution), and maintained by additional doses of 1mL of a 40%w/v solution as required.

In summary, the surgery performed on the animals comprised: a tracheotomy to maintain respiratory function during anaesthesia; isolation and ligation of the oesophagus to prevent drainage of any intranasally administered drug into the gut; and cannulation of the jugular vein for collection of blood samples.

Each anaesthetised rat was laid ventral side uppermost and an incision was made in the skin over the trachea exposing the underlying muscle. The muscle layer over the trachea was carefully dissected and a transverse incision made in the upper surface of the trachea. A cannula (3.0mm o.d.; 2.0mm i.d.) was inserted through the incision and into the trachea. This cannula was secured with thread. The oesophagus was isolated and ligated using thread.

Using blunt dissection, the jugular vein on one side of the neck was exposed and ligated distal to the heart. An incision was made in the vein, midway along the exposed section, between the ligature and the heart, and a cannula (1.0mm o.d.; 0.5mm i.d.) was inserted and secured using a ligature. The cannula was kept free from clots by flushing with a saline solution containing heparin (25 U mL⁻¹). After removing each blood sample the cannula was flushed through with a small volume of heparinised saline.

6.3.4 Drug Administration

After the surgery described in section 6.3.2 had been performed, each animal was laid ventral side uppermost on a heated work surface to prevent hypothermia during anaesthesia. A 100µl Hamilton syringe was filled via a length of polypropylene tubing (0.8mm o.d.; 0.4mm i.d.) attached to the needle, with 50µl of the dose solution/suspension. The tubing was inserted approximately 2mm into the rat nostril, and the dose administered as a bolus (≤ 30 seconds to dose). At time zero, each rat received equivalent to 1mg of propranolol in solution/suspension in a 50µl volume, delivered as a single dose into one nostril.

6.3.5 Sample Collection

Blood samples (100µl) were withdrawn from the jugular vein cannula and collected into heparinised tubes at 2, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 minutes after administration.

Plasma was collected by centrifuging samples at 300 r.p.m. for 2 minutes.

For dose group 3, blood samples were only collected until 120 minutes after drug administration. This was because the rats in this group had not been starved prior to administration of urethane, and the resultant increase in absorption of the anaesthetic proved fatal to the rats after 120 minutes.

6.3.6 Spectrofluorimetric Assay for Plasma Propranolol

Plasma concentrations of propranolol were determined by a spectrofluorimetric assay developed by Critchley (1989). This was modified from a technique described by Shand et al (1970) who reported that the assay is free from interference by metabolites of propranolol.

Fluorescence measurements were made on a Perkin-Elmer 3000 Fluorescence Spectrometer using a 1cm path length quartz cell. The maximum excitation and emission wavelengths of propranolol in 0.1M HCl were found to be 285nm. and 345nm. respectively. A slit width of 5nm. was used for both the excitation and emission wavelengths. Measurements were taken as the mean of five successive readings.

To remove contaminants which could result in background fluorescence, all glassware was surface cleaned with chromic acid and rinsed five times in tap water and five times in distilled water prior to use.

6.3.7 Standard Curve

In order to allow a quantitative determination of propranolol concentration in unknown samples, a standard curve of fluorescence vs. propranolol concentration was determined on the day of each assay. Standard solutions of propranolol in 0.1M HCl (2.5, 5, 10, 20, 40, 50 and 100 ng mL⁻¹) were prepared by dilution of a 0.1mg mL⁻¹

stock solution. The fluorescence of each standard was measured in triplicate, and a standard curve of fluorescence vs. propranolol concentration was constructed. This was found to be linear over a range of concentrations. A typical standard curve is illustrated in Figure 6.1.

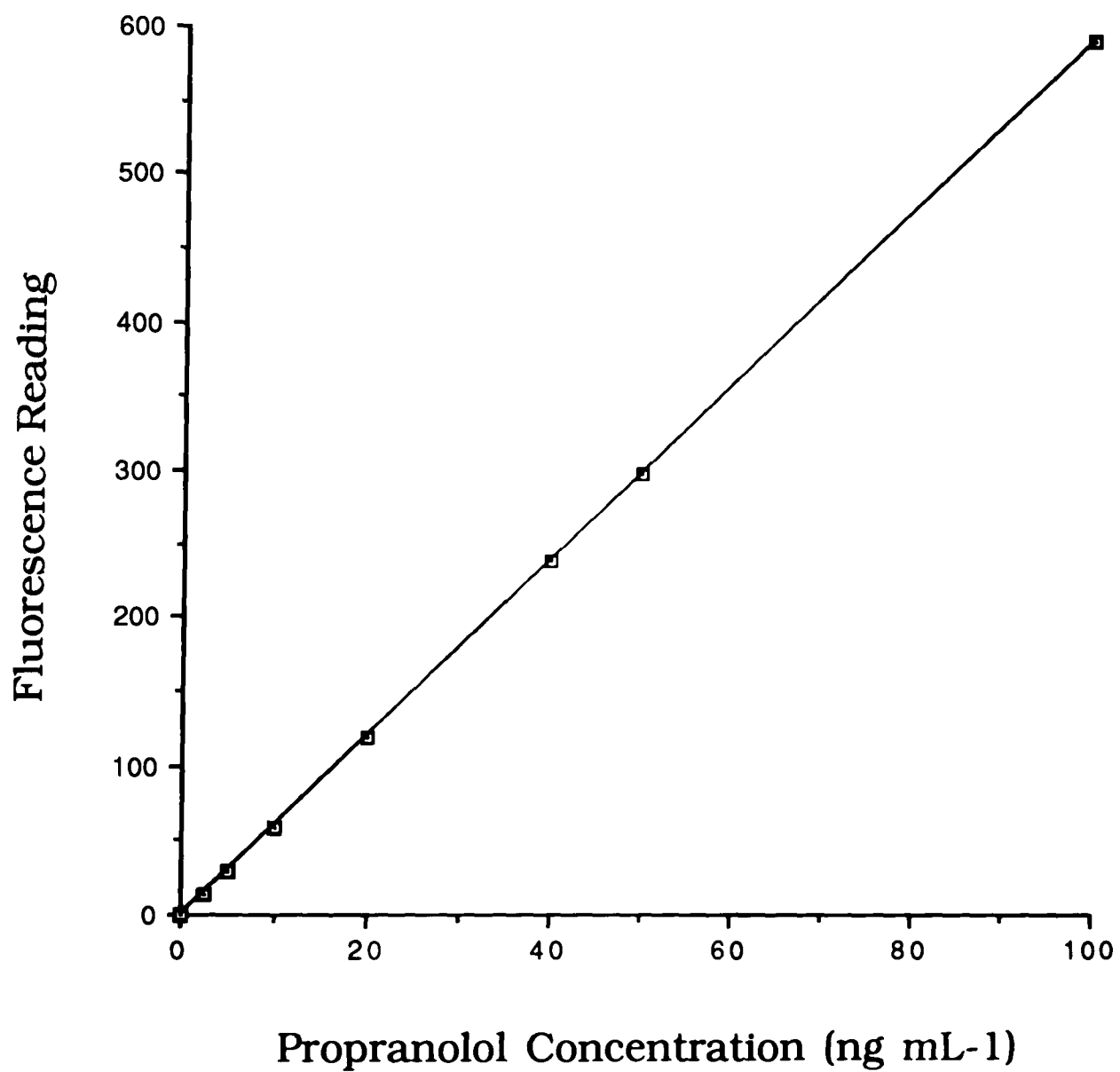
6.3.8 Extraction of Drug from Plasma

All the plasma samples were extracted prior to spectrofluorimetric assay. After storage in a freezer at -20°C they were thawed at room temperature. 250µl of distilled water was added to 50µl of plasma in a glass test tube and made alkaline by the addition of 250µl of 1M NaOH. This mixture was extracted into 3mL of iso-amyl alcohol (1.5%) in heptane, by vortexing for three minutes and centrifuging for 10 minutes at 3000 r.p.m. (The iso-amyl alcohol in heptane was added as 50µL iso-amyl alcohol and 2.95mL heptane due to variable extraction efficiencies when a stock mixture was used). 2mL of the heptane layer was removed and placed in a clean test tube. 3mL of 0.1M hydrochloric acid was added and the mixture vortexed for 3 minutes, then centrifuged as before (3000 r.p.m. for 10 minutes). The upper organic phase was removed and the acid layer assayed spectrofluorimetrically for propranolol using 0.1M HCl as the blank.

6.3.9 Efficiency/Reproducibility of the Extraction Procedure

Blank rat plasma (100µl) was spiked with a known amount of propranolol, then extracted and assayed as outlined above. The typical extraction efficiency was found to be $90 \pm 1.2\%$, therefore this correction factor was applied to all future samples assayed.

Figure 6.1
Typical Standard Curve of Fluorescence
versus Concentration for Propranolol



6.4 PHARMACOKINETIC PARAMETERS

6.4.1 Area Under The Curve (AUC)

The area under the plasma time curve (AUC) following IV or IN administration reflects the amount of drug absorbed systemically and can be determined by numerical integration from $t=0$ to $t=\infty$.

The AUC was calculated for each of the three dosage forms above using a computer program written by Dr. P. J. Kirk (Department of Physics, University of Nottingham). This program calculates a minimum value taking the last point of the curve (that is, time = 240 minutes for Figure 6.2) as the cut-off point, and a theoretical maximum value (curve extrapolated to zero concentration using a least-squared fit procedure on the last five data points) for area under the curve. It was tested using measurements from Hussain et al (1980b) and was found to generate results in agreement with these workers. The program is listed in Appendix 2.

6.4.2 Absolute Bioavailability (F%)

Bioavailability may be defined as the rate and extent to which drug is absorbed and becomes available to the systemic circulation (Wagner 1975). Therefore, it is a useful parameter for comparing drug absorption from different formulations administered via the same route.

IV administration is considered to result in complete bioavailability since drug is introduced directly into the systemic circulation. Thus, absolute bioavailability (F%) may be calculated by comparing AUC data obtained following IV dosing with AUC data from extravascular dosing:

$$F\% = \frac{AUC_{0-\infty \text{ e.v.}}}{AUC_{0-\infty \text{ i.v.}}} \times \frac{\text{Dose}_{\text{ i.v.}}}{\text{Dose}_{\text{ e.v.}}} \times 100 \quad (\text{Equation 6.1})$$

where: AUC = area under the plasma time curve;
 Dose_{i.v.} = intravenous dose;
 Dose_{e.v.} = extravascular dose.

6.4.3 Relative Bioavailability (F%_{rel})

The determination of absolute bioavailability requires both IV and extravascular administration of a drug. However, relative bioavailability (F%_{rel}) can be employed to compare different formulations of the same drug administered extravascularly, and may be calculated using equation 6.2:

$$F\%_{\text{rel}} = \frac{AUC_{0-\infty \text{ e.v.}(1)}}{AUC_{0-\infty \text{ e.v.}(2)}} \times \frac{\text{Dose}_{\text{ e.v.}(2)}}{\text{Dose}_{\text{ e.v.}(1)}} \times 100 \quad (\text{Equation 6.2})$$

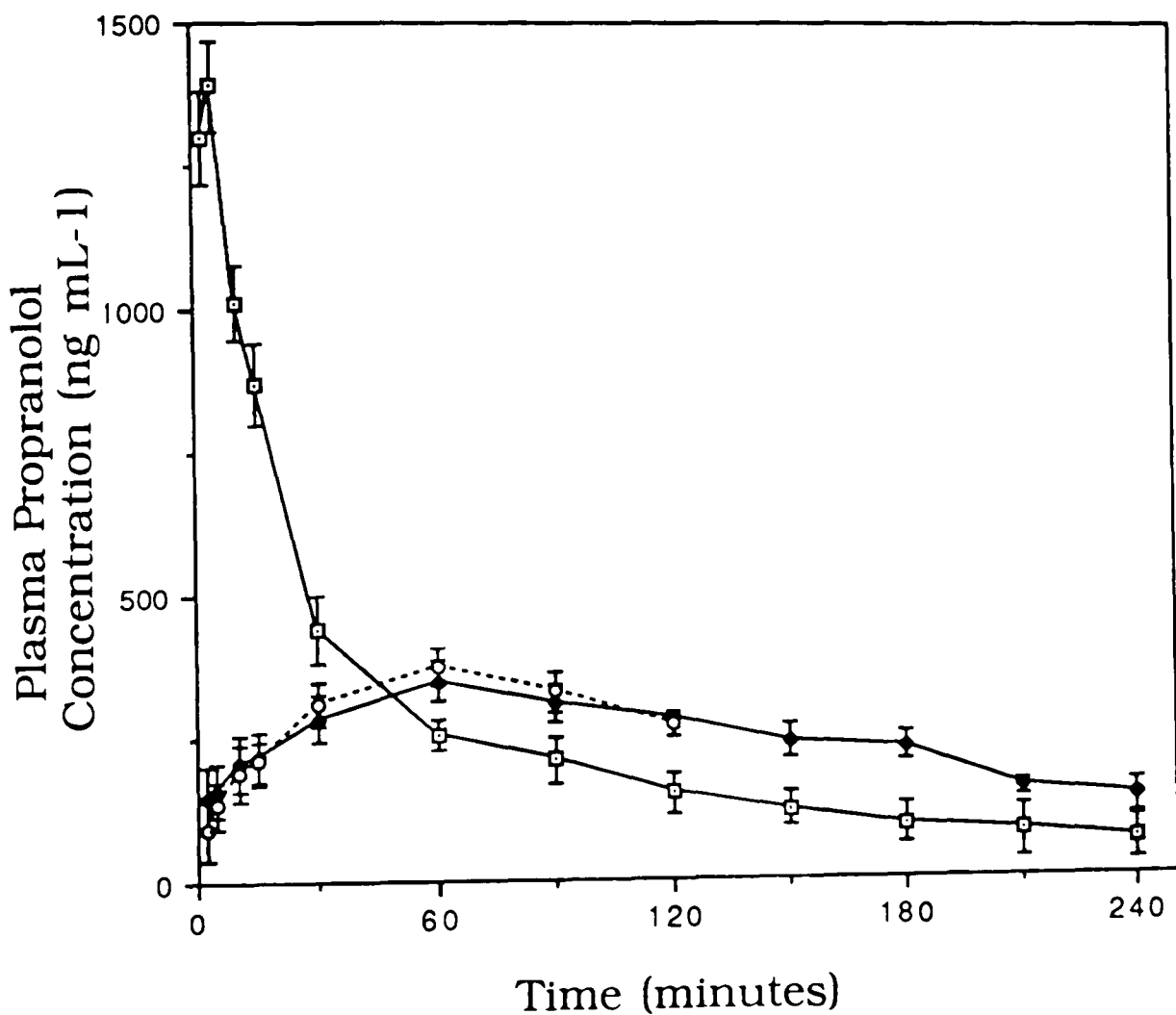
6.5 RESULTS

Mean plasma concentration versus time profiles for the formulations of propranolol outlined in section 6.3.1 are shown in Figure 6.2.

It can be seen from the results for Dose Group 1 (propranolol solution) that the absorption of propranolol from the nasal cavity of rats was rapid, resulting in a sharp peak in plasma levels 5 minutes after administration, followed by a rapid decline in the plasma concentration/time curve.

Administration of propranolol as an alginate complex (Dose Group 2) resulted in a significant change in the rate of drug

Figure 6.2
 Blood Plasma Profiles after Nasal Delivery
 of Propranolol Formulations to Rats



—□— Dose group 1 - propranolol solution

—●— Dose group 2 - propranolol/alginate complex
 in deionised water

.....○..... Dose group 3 - propranolol/alginate complex in a
 formulation made isotonic with glycerol

absorption compared to the solution formulation. For the complex, the absorption rate was much slower with peak plasma levels being achieved after 60 minutes, but was sustained over a greater period of time.

The presence of glycerol to adjust tonicity (Dose Group 3) did not appear to markedly change the absorption profile for the propranolol/alginate complex in rats, in comparison with Dose Group 2.

6.5.1 Area Under the Curve (AUC)

The areas under the curves for each of the dosage forms were calculated using the computer program in Appendix 2 as follows:

(i) Dosage Form 1 (Propranolol solution):

AUC : Minimum = 990;

Maximum = 1047 ± 2 .

(ii) Dosage Form 2 (Alginate/propranolol complex in deionised water):

AUC : Minimum = 987;

Maximum = 1113 ± 10 .

(iii) Dosage Form 3 (Alginate/propranolol complex in isotonic glycerol solution):

The area under this curve could not be compared to the values for (i) and (ii) above, as data was only available up to two hours into the experiment (section 6.3.4), compared to four hours for the above. However, it can be seen from Figure 6.2 that curve (iii) followed a very similar pattern to curve (ii), with peak plasma levels occurring at 60 minutes after administration, therefore provided that this trend continued, which seems likely, it can be assumed that, in rats, the presence of glycerol does not affect the rate of absorption of propranolol from the propranolol/alginate complex.

Table 6.1 lists the pharmacokinetic parameters AUC and Relative Bioavailability described above, calculated for Dose Groups 1 and 2 using equation 6.2.

Table 6.1 Pharmacokinetic Parameters after Nasal Administration of Propranolol to Rats

<u>Formulation</u>	<u>AUC_{0-∞}</u> <u>(ng hr) mL⁻¹</u>	<u>Dose</u> <u>(mg)</u>	<u>Relative</u> <u>Bioavailability</u> <u>(%)</u>
Solution (Dose Group 1)	1047 ± 2	1	100
Sustained release (Dose Group 2)	1113 ± 10	1.1	97

The results for Dose Group 1 (propranolol solution) correspond well with previously published results for nasal absorption of propranolol in rats (Hussain et al 1980b). These workers reported a nasal bioavailability of 99.9% compared to that of an IV dose.

6.5.2 Relative Bioavailability

A comparison of the results for relative bioavailability from Table 6.1 indicates that there is little difference in the bioavailability of propranolol delivered nasally to rats as a solution (Dose Group 1) or as a complex with alginate (Dose Group 2).

6.5.3 Absolute Bioavailability

Hussain et al (1980b) have published bioavailability data following IV administration of propranolol to rats. Their experiments were performed using the same dose of propranolol in rats of a similar size to those employed in these investigations, thereby allowing a comparison with the bioavailabilities for

Dose Groups 1 and 2 to be made with reasonable confidence. The AUC calculated by Hussain et al (1980) for IV administration of propranolol was $1033.8 \pm 30.8 \text{ ng hr mL}^{-1}$.

The results for Dose Group 1 (absolute bioavailability = $101 \pm 3\%$, calculated using equation 6.1) are in agreement with Hussain et al (1980b), who reported an absolute bioavailability of 100% for propranolol solution delivered IN, compared to an IV formulation. In comparison with the IV results published by Hussain et al, for the formulation containing the propranolol/alginate complex, the absolute bioavailability is $98 \pm 3\%$.

6.6 DISCUSSION

From the results above it can be seen that, by complexing propranolol with alginate, it is possible to control the rapid release of propranolol usually observed after nasal delivery of the drug in solution, whilst still retaining a systemic bioavailability comparable to that of the solution. The use of a sustained release formulation based on alginate complexation may have important advantages for drugs such as propranolol which are rapidly absorbed via the nasal route. For example, the results in rats indicate that it may be possible to eliminate the 'peaks and troughs' in plasma drug concentration which are often observed with conventional dosage forms. In humans, this may markedly reduce the incidence of unwanted systemic or local side-effects and could thereby improve patient compliance.

It is postulated that the mechanism of release of propranolol from the complex in the nasal cavity may follow the pattern demonstrated *in vitro* in Chapter 5. The alginate and propranolol are administered intranasally as an ionic complex suspended in deionised water. Cations, including sodium and calcium, which are present in the rat nasal fluids (Chien and Chang 1987) cause the propranolol/alginate complex to dissociate as the propranolol on the alginate is slowly ion-exchanged by these cations. The *in vitro*

experiments (Chapter 5) did not suggest that propranolol release from the complex would be sustained for the relatively long period of time observed in the *in vivo* experiments. From the *in vitro* experiments, it was thought that once the insoluble complex had dissolved by formation of soluble sodium alginate, absorption of propranolol *in vivo* would follow the rapid pattern normally observed with administration of propranolol solution. However, the sustained release observed *in vivo* may be due to other factors. For example, whilst ions such as calcium may displace propranolol from the propranolol/alginate complex, they may also cause the alginate to gel, thereby delaying the absorption of free propranolol, since it would have to diffuse through the gel network before being absorbed through the rat nasal mucosa. Mucus in the nasal cavity may also act as a barrier to the absorption of propranolol, compared to the results observed *in vitro* when no mucus was present.

The mechanism by which drugs are absorbed across the nasal mucosa is not yet fully understood. The pH of the nasal cavity has been quoted as 7.2-7.4 (Hirai et al 1981), which would suggest that the ionised form of propranolol would predominate (pKa propranolol = 9.45). However, there must be sufficient of the unionised form present to allow good absorption to take place, since the absorption of propranolol has been found to be both rapid and dose-dependent (Critchley 1989). In addition, propranolol is a relatively small and lipophilic drug (MW 295; log P octanol/water 3.36), thus Hirai et al (1981) indicate that the most likely mechanism for its absorption is by transcellular diffusion in accordance with the pH/partition hypothesis.

The anaesthetised rat is useful for preliminary investigations of the absorption characteristics of nasally delivered propranolol. However, as discussed in section 6.1.4, it cannot be totally representative of the physiological situation in man, since there may be differences in absorption due to anaesthesia, surgical intervention or orientation of the rat. For example, the animals were anaesthetised with urethane. Propranolol is known to be

predominantly eliminated from the body by the liver and the lung (Frishman 1979). Thus, the pharmacological effects of the anaesthetic which include lowering of basal metabolic rate and changes in hepatic function may result in a difference in nasal absorption between conscious and anaesthetised animals. Also, during surgery, the oesophagus was tied off to prevent drainage of intranasally administered propranolol into the gut. This would not be representative of the case in a conscious human subject, where propranolol could drain freely into the gastrointestinal tract. Finally, in these studies, the animals were dosed while lying horizontally on their backs. This could lead to dose pooling over the upper regions of the nasal cavity, and cannot therefore be directly compared to the situation when a man is dosed in an upright vertical position.

Despite the favourable results reported in these experiments and by Hussain et al (1980b), there is a drawback to the nasal administration of propranolol. The nasal ciliary epithelium plays an important role in the removal of dust, allergens and micro-organisms which may be deposited in the nose during inhalation. Van de Donk and Merkus (1982) investigated the chronic effect of long-term IN administration of propranolol on the ciliary beat frequency in chicken embryo and human adenoid tissue. Their results demonstrated that propranolol in nose drops arrested the movement of chicken and human cilia within twenty minutes of administration of doses of 1% and 0.1% respectively. Another study reported a significant reduction in mucociliary clearance after oral administration of propranolol to humans. This toxic effect of propranolol has also been found with other cilia-like structures, for example the flagella of human spermatozoae (Zipper et al 1983). For these reasons, propranolol is unacceptable for long-term therapy if delivered intranasally.

However, in these experiments propranolol was employed only as a model cationic lipophilic drug. The results have demonstrated that the complexation of this cationic drug with an alginate results in more sustained release rates from the complex

than from a drug solution alone. That is, it may be possible to slow down and sustain the release of drugs which are rapidly absorbed through the nose, without affecting the bioavailability of these drugs. This may prove to have important consequences for a variety of other substances which could be delivered intranasally.

6.7 CONCLUSIONS

The nasal delivery to rats of a propranolol/alginate complex resulted in a significant reduction in the rate of drug absorption compared to a solution formulation. The rate of absorption of propranolol from the complex was much slower and was sustained over a greater period of time, compared with absorption from solution.

The systemic bioavailability of the drug from the complex was comparable to that of the solution, and to that of an IV dose carried out in rats by other workers (Hussain et al 1980b).

It is possible that the results reported above for the model drug propranolol and the polysaccharide sodium alginate may be applicable to other drug/polysaccharide systems, which could be used in the future to develop sustained release delivery systems for use in humans. It would therefore be of benefit to continue these investigations using other combinations of drugs and polysaccharides.

CHAPTER 7 SUMMARY

The aim of the work in this thesis was to achieve a greater understanding of the fundamental mechanisms and factors controlling the interaction of an anionic polysaccharide polymer, sodium alginate, with a cationic drug molecule in an aqueous environment, and to apply this knowledge to provide a rational approach to the design of a controlled release drug delivery system based on drug:polysaccharide complexes.

To date, the interaction of ionic polysaccharides with drug molecules has been viewed as a disadvantage, for example, it has been reported that cationic antiseptics, including acriflavine chloride and cetylpyridinium chloride become inactivated in 1% ammonium alginate gel as a result of complex formation (Keipert et al 1973). There have been few studies which have investigated the potential of using such interactions for controlling drug delivery, even though the control of drug release is desirable in many routes of administration (for example, the nasal route) in order to prevent peaks and troughs in blood plasma levels. The nasal route in the rat was chosen as a suitable animal model in which to test this system, since many drugs are readily absorbed from the nasal cavity, but with some, absorption occurs so rapidly that the resulting high blood level peaks may give rise to side-effects. The model drug propranolol hydrochloride is rapidly absorbed intranasally, therefore control of drug release is required to retard absorption of drug and to flatten the blood level peaks.

Polymers are routinely included in nasal liquid formulations to prolong nasal retention time by increasing viscosity, and some ionic polysaccharides appear to slow the release of certain nasally administered drugs over and above that seen from a viscosity effect alone. It was therefore decided to investigate the interaction of an ionic polysaccharide, sodium alginate, with a model cationic drug, propranolol hydrochloride, with a view to designing a controlled release drug delivery system.

In these studies, two highly purified alginates from *Laminaria hyperborea* and *Ascophyllum nodosum* were first characterised in terms of molecular weight, polydispersity and M:G ratio, prior to drug binding studies to investigate their interaction with propranolol hydrochloride. The *Laminaria hyperborea* alginate was found to have a higher proportion of G-blocks (M:G ratio = 35:65) than the *Ascophyllum nodosum* alginate (M:G ratio = 61:39).

Preliminary investigations on the interaction between sodium alginate and propranolol using viscometry and nephelometry provided evidence that, above certain concentrations, there was an interaction between propranolol and sodium alginate in deionised water resulting in the formation of a precipitate. Furthermore, it was found that the addition of sufficient counter-ions, for example sodium chloride ions, could re-dissolve the precipitate, suggesting that the interaction was ionic in nature, possibly as a result of ion-pairing between the positively-charged propranolol and the negatively-charged alginate.

In order to quantify this interaction in the presence and absence of sodium chloride, an equilibrium dialysis technique was used. The results from the dialysis studies again suggested that the interaction between alginate and propranolol was ionic in nature and that at the saturation point, stoichiometric 'one-to-one' binding was occurring, with one propranolol molecule interacting with one carboxyl group on each hexose residue of the alginate chain. No difference in the degree of binding of propranolol to the high-G or high-M alginate was observed. This was unexpected, since it has been found by other workers that with, for example, calcium ions, the interaction is much stronger with high-G alginates and results in the formation of strong brittle gels compared with the much weaker gels formed with high-M alginates. Examination of the binding isotherms indicated that 'negative co-operativity' was occurring, so that the binding of one propranolol molecule to a carboxyl group on the alginate made it more difficult for other propranolol molecules to bind to adjacent carboxyl groups. The use of molecular modelling

to visualise the possible *in-vacuo* three-dimensional structure of the ionic complex formed between propranolol and alginate supported this finding, in that it showed that the presence of one propranolol molecule binding to the alginate could make it stereochemically difficult for subsequent molecules to bind.

In order to prepare a dosage form which could be conveniently reconstituted for administration *in vivo*, a freeze-dried alginate/propranolol complex was prepared. This was suspended in either deionised water or in isotonic glycerol solution and tested *in vitro* for release of propranolol across a semi-permeable membrane into sodium chloride solution. It was found that release of propranolol from the complex in suspension was delayed compared with release from a propranolol solution. It is postulated that the reason for this delay is that, whereas the release profile for propranolol solution is dependent only on the rate of diffusion across the semi-permeable membrane, several processes occur in order for propranolol to be released from the complex. That is, sodium ions diffuse in and, since the affinity of the sodium ions for the alginate is greater than that of the propranolol ions, ion exchange occurs releasing drug and dissolving the propranolol/alginate complex. Propranolol ions then diffuse across the membrane into the sodium chloride solution.

The release of propranolol from the formulations previously tested *in vitro* was then assessed *in vivo*. Nasal delivery in the rat was chosen as a model *in vivo* system in which to investigate the potential of alginate ionic complexes to control drug absorption. It was found that the nasal delivery to rats of a propranolol/alginate complex in suspension resulted in a significant reduction in the rate of drug absorption compared to a solution formulation, but that the bioavailability remained unchanged and comparable to that of an IV dose carried out in rats by other workers (Hussain et al 1980b). The sustained release observed *in vivo* was much greater than would have been predicted from the *in vitro* results. However, this may be due to several factors. For example, there is a limited amount of fluid

present in the nose of the rat compared to the almost 'sink' conditions employed in the *in vitro* experiments. In addition, mucus in the nose could act as a barrier to the *in vivo* absorption of propranolol. Lastly, although the presence of cations such as Ca^{2+} in the mucus could displace propranolol from the complex, they could also cause the alginate to gel, thereby delaying propranolol absorption since it would have to first diffuse through the polymer gel network.

The work in this thesis has focussed on the interaction and complexation of one ionic polysaccharide with a model drug. The results have demonstrated that, *in vivo* via the nasal route, this complexation results in more sustained release rates from the complex than from a drug solution alone, without affecting the bioavailability of the drug. It is possible that these results may be applicable to a variety of other naturally occurring or synthetic polysaccharides combined with drugs which have applications for nasal delivery (either for their local or their systemic effects). In addition, the complexation of drugs with polysaccharides may have potential as delivery systems for many other routes, for example, the oral, vaginal rectal and ocular routes, or in wound dressings and dental pastes.

The future success of nasal delivery systems for humans depends on their being commercially viable compared to, for example, parenteral or oral administration. Therefore, future work should include systematic investigations into the interaction of a range of polysaccharides with various drugs in order to manipulate the physicochemical properties (and thereby the absorption characteristics) of the drug molecules. Such investigations may allow the development of novel delivery systems which in the future could prove to have important consequences for the delivery of a variety of drugs, for example insulin for diabetic patients, which at present can only be administered via the parenteral route. Who nose?

APPENDIX 1

Calculation of the Composition and Sequence Parameters from the NMR Spectra of *L. hyperborea* and *A. nodosum* alginates.

The following equations apply to the NMR spectra shown in figures 2.9 and 2.10 (I is the area under the peaks):

$$F_G = F_{GG} + F_{GM}$$

$$F_{GG} = F_{GGG} + F_{GGM}$$

$$I_{\text{total}} = I_G + I_{MM} + I_{MG}$$

$$\text{(where } I_{MG} = (I_{MG} + I_{MGG} + I_{MGM})/2 \text{)}$$

$$F_G = I_G / I_{\text{total}}$$

$$F_M = 1 - F_G$$

$$F_{GG} = I_{GG} / I_{\text{total}}$$

$$F_{MG,GM} = F_G - F_{GG}$$

$$F_{MM} = F_M - F_{MG,MG}$$

$$F_{GGG} = I_{GGG} / I_{\text{total}}$$

$$F_{GGM} = F_{GG} - F_{GGG}$$

$$F_{MGM} = F_{GM} - F_{GGM}$$

$$N_G \text{ (average length of G-blocks)} = F_G / F_{MG}$$

(i) Laminaria hyperborea alginata

$$I_{\text{total}} = 1144 + 401 + (213 + 90 + 146)/2 = \underline{1769.5}$$

$$F_G = 1144/1769.5 = \underline{0.647}$$

$$F_M = 1 - 0.647 = \underline{0.353}$$

$$F_{GG} = (832 + 129)/1769.5 = \underline{0.543}$$

$$F_{MG,GM} = 0.647 - 0.543 = \underline{0.104}$$

$$F_{MM} = 0.353 - 0.104 = \underline{0.249}$$

$$F_{GGG} = 832/1769.5 = \underline{0.470}$$

$$F_{GGM} = 0.543 - 0.470 = \underline{0.073}$$

$$F_{MGM} = 0.104 - 0.073 = \underline{0.031}$$

$$N_G = 0.647/0.104 = \underline{6.2}$$

(ii) Ascophyllum nodosum alginata

$$I_{\text{total}} = 527 + 547 + (245 + 95 + 221)/2 = \underline{1354.5}$$

$$F_G = 527/1354.5 = \underline{0.389}$$

$$F_M = 1 - 0.389 = \underline{0.611}$$

$$F_{GG} = (228 + 89)/1345.5 = \underline{0.234}$$

$$F_{MG,GM} = 0.389 - 0.234 = \underline{0.155}$$

$$F_{MM} = 0.611 - 0.155 = \underline{0.456}$$

$$F_{GGG} = 228/1354.5 = \underline{0.168}$$

$$F_{GGM} = 0.234 - 0.168 = \underline{0.066}$$

$$F_{MGM} = 0.155 - 0.066 = \underline{0.089}$$

$$N_G = 0.389/0.155 = \underline{2.5}$$

APPENDIX 2

Program to Calculate Area under the Plasma/Time Curve (AUC)

```
1 REM *****
2 REM
3 REM          INTEGRAL
4 REM
5 REM *****
10 REM *****
20 REM This program calculates the area under a curve
30 REM of a set of data points
40 REM An extrapolation to zero is then carried out
45 REM using a least square fit procedure
50 REM to find the area under the tail
60 REM Dr P J Kirk
65 REM Department of Physics, University of Nottingham
70 REM
80 REM *****
100 REM The variable X(I) represents the TIME variable
110 REM The variable Y(I) represents the concentration
120 REM of propranolol at time t
200 REM *****Input data*****
210 DIM X(100),Y(100)
220 CLS
230 WRITE
240 INPUT "Please input the number of data points(5-100)";NP
243 IF (NP<5) OR (NP>100) THEN PRINT "TRY AGAIN" :GOTO 240
245 PRINT "Please input the data points in ascending values of x"
250 FOR I = 1 TO NP
255 PRINT "Data point ";I
260 INPUT "Please input the TIME (in minutes)";X(I)
270 INPUT "          the Prop CONC";Y(I)
280 NEXT I
290 GOSUB 1000
300 REM ***** work out linear regression on last five points***
310 REM***** convert X(I) into hours *****
315 FOR I = 1 TO NP : X(I) = X(I)/60:NEXT I
320 XSUM = 0!
321 YSUM =0!
325 N = 4
330 FOR I = (NP-N+1) TO NP
340 XSUM = XSUM + X(I)
350 YSUM = YSUM +Y(I)
360 NEXT I
370 XMEAN = XSUM/N
380 YMEAN = YSUM/N
390 REM *** Find the best fit gradient (A) and best fit intercept (B)**
400 M = 0 : D = 0 :A =0
410 FOR I = (NP-N +1) TO NP
420 M = M + ( Y(I)*(X(I)-XMEAN))
430 D = D + ((X(I)-XMEAN)^2)
435 NEXT I
440 A = M/D
450 B = YMEAN - A*XMEAN
460 M=0
470 FOR I = (NP-N +1) TO NP
480 M = M + ((Y(I)-A*X(I)-B)^2)
490 NEXT I
500 DA = M /((N-2)*D)
505 DA = SQR(DA)
510 DB = ( (1/N) + ( XMEAN^2)/D))*(M/(N-2))
515 DB = SQR(DB)
520 REM ***** end of regression *****
530 REM ***** find x axis intercept *****
535 IF (A =0) THEN PRINT" REGRESSION HORIZONTAL":END
540 X(NP+1) = -B/A
550 Y(NP+1) =0
```



```

600 REM *** Calculuate area under the curve *****
605 REM *** using the trapezium rule *****
610 AREA1 = 0!
620 AREA2=0!
630 FOR I = 1 TO NP-1
640 AREA1 = AREA1 + ( X(I+1) -X(I))*(Y(I+1) +Y(I))/2
650 NEXT I
660 AREA2 = AREA1 + ( X(NP+1)-X(NP))*(Y(NP+1)+Y(NP))/2
670 DELA = ABS((AREA2-AREA1)*( (DB/B) + (DA/A)))
700 REM ***** Print results *****
710 CLS
720 FOR I = 1 TO 5 : PRINT " " : NEXT I
730 PRINT "          The minimum area without extrapolation = ";AREA1
740 PRINT
750 PRINT "          The area with Linear extrapolation = ";AREA2
755 PRINT "          The error = +/-";DELA
756 FOR I = 1 TO 3 :PRINT"":NEXT I
760 PRINT ," R   Run Integral With a New Set Of Data"
765 PRINT ," M   Modify Data and Re - Run Integral "
775 PRINT ," E   End "
780 BS = INKEYS
790 IF LEN(BS) = 0 THEN GOTO 780
800 IF (ASC(BS)=82 OR ASC(BS) = 114)THEN GOTO 10
820 IF (ASC(BS)=77 OR ASC(BS) = 109)THEN GOSUB 1000:GOTO 300
830 IF (ASC(BS)=69 OR ASC(BS) = 101)THEN GOTO 999
999 END
1000 REM ***** This subroutine checks the data and allows *****
1001 REM ***** changes to the data before integration *****
1010 CLS
1020 PRINT" "
1030 PRINT "" ,"I", "Time(mins)", "Conc"
1040 PRINT
1050 FOR I = 1 TO NP
1060 PRINT "" ,I,X(I),Y(I)
1070 NEXT I
1075 PRINT " Do you wish to change any data?(Y/N)"
1080 AS = INKEYS
1085 IF LEN(AS) = 0 THEN 1080
1090 IF (ASC(AS) =110 OR ASC(AS) = 78 ) THEN GOTO 1200
1100 IF (ASC(AS) = 121 OR ASC(AS) = 89 ) THEN GOTO 1120
1110 GOTO 1080
1120 INPUT " Please input number of data set you wish to change";C
1130 PRINT ""
1140 INPUT "Please input the new TIME ";X(C)
1150 INPUT "          new CONC ";Y(C)
1160 GOTO 1010
1200 RETURN

```

BIBLIOGRAPHY

Abraham, R.J. and Smith, P.E. **Nucleic Acid Res.** 1988; 16: 2639.

Abraham, R.J. and Smith, P.E. **J Comput. Chem.** 1988; 9: 288.

Agfa, A.G. 1964; **British Patent** 962483.

Ak, M.M., Nussinovitch, O.H., Campanella, O.H. and Peleg, M.
Biotechnology Progress 1989; 5 : 75-77.

Albery, W.J., Burke, J.F., Leffler, E.B. and Hadgraft, J. **J. Chem. Soc. Faraday Trans. 1** 1970; 72 : 1618-1626.

Alonso, C.M., Bernabé, R.R. and Moreno, E. **J. Immunol. Methods** 1978; **22** : 361-368.

Anderson, M.T., Harding, S.E. and Davis, S.S. **Biochem. Soc Trans.** 1989; 17 : 1101-1102.

Andrews, P. **Biochem. J.** 1965; 96 : 595-606.

Arneodo, C. and Benoit, J.P. **Polym. Mater. Sci. Eng.** 1987; 57 : 255-259.

Ashley, J.J. and Levy, G. **J. Pharm. Sci.** 1973; 62 : 688-690.

Atkins, E.D.T., Mackie, W., Parker, K.D. and Smolko, E.E.
J. Polymer Sci. B : Polymer Letters 1971; 9 : 311-316.

Atkins, E.D.T., Nieduszynski, I.A., Mackie, W., Parker, K.D. and Smolko, E.E. **Biopolymers** 1973a; 12 : 1865-1878.

Atkins, E.D.T., Nieduszynski, I.A., Mackie, W., Parker, K.D. and Smolko, E.E. **Biopolymers** 1973b; 12 : 1879-1887.

- Badwan, A.A., Abumalooch, A., Sallam, E., Abukalaf, A. and Jawan, O. **Drug Del. Ind. Pharm.** 1985; 11 : 239-256.
- Bailey E., Mitchell, J.R. and Blanshard, J.M.V. **Colloid and Polymer Sci.** 1977; 255 : 856-860.
- Ball, A., Harding, S.E. and Mitchell, J.R. **Int. J. Biol. Macromol.** 1988; 10 : 259-264.
- Becirevic, M. and Petricic, V. **Acta Chem. Jugoslav.** 1980; 30 : 109-112.
- Beldie, C., Dumitriu, S., Aelenei, N., Popa, M. and Dumitriu, D. **Biomaterials** 1989; 10 : 622-624.
- Berger, F.M. et al. **Am. J. Digestive Diseases** 1953; 20: 39.
- Bernal, V.M., Smajda, C.H., Smith, J.L. and Stanley, D.W. **J. Food Sci.** 1987; 52 : 1121-1136.
- Berner, L.A. and Hood, L.F. **J. Food. Sci.** 1983; 48 : 755-758.
- Blair, S.D., Jarvis, P., Salmon, M. and McCollum, C. **Br. J. Surg.** 1990; 77 : 568-570.
- Blumgart, H.L. **Arch. Intern. Med.** 1922; 29 : 508-514.
- Bock, K. **Pure and Appl. Chem** 1987; 59 : 1447-1456.
- Bodmeier, R. and Paeratakul, O. **J. Pharm Sci.** 1989; 78 : 964-967.
- Bowman, W.C. and Rand, M.J. (eds.), **Textbook of Pharmacology.** 2nd edn. 1980; Blackwell Scientific Publications, Oxford.

Boyd, J. and Turvey, J.R. **Carbohydr. Res.** 1978; 66 : 187-194.

British National Formulary Number 24 (September 1992); British Medical Association and Royal Pharmaceutical Society of Great Britain.

British Pharmacopoeia 1988, Vols. 1 and 2, HMSO, London.

Burger, J.C. **Prog. Fd. Nutr. Sci.** 1982; 6 : 235-246.

Carr, C.W. 1961; in **Physical Methods in Chemical Analysis IV**, ed. Berl, W.G., Academic Press, New York.

Carr, J.E.F. and Nolan, J. **Nature** (London) 1968; 219 : 500-501.

Carr, J.E.F., Nolan, J. and Durakovic, A. **Nature** (London) 1969; 224 : 1115.

Ceri, H., McArthur, H.A.I. and Whitfield, C. **Infection and Immunity** 1986; 51 : 1-5.

Césaro, A., Delben, F. and Paoletti, S. **J. Chem. Soc., Faraday Trans. 1** 1988; 84 : 2573-2584.

Chien, Y.W. (Ed.) **Transnasal Systemic Medications**, 1985; Elsevier, New York.

Chien, Y.W. and Chang, S.-F. **CRC Crit. Rev. in Ther. Drug Carr. Sys.** 1987; 4 : 1-194.

Clark, K.P. and Falk, R.A. **European Pat. Appl.**, E.P. 311570 A2, 12th April 1989.

Cohen, M.B., Ecker, E.E., Breitbart, J.R. and Rudolph, J.A. **J. Immunol.** 1930; 18 : 419-425.

- Cottrell, I.W. and Kovacs, P. 1977; in **Food Colloids**, ed. Graham H.D., Avi Publishing Co. Inc., Connecticut.
- Creeth, J.M. and Harding, S.E. **J. Biochem. Biophys. Methods** 1982; 7 : 25-34.
- Crescenzi, V. and Brittain, H.G. **J. Polym. Sci. : Polymer Physics Edition** 1985; 23 : 437-443.
- Critchley, H. 1989 **Intranasal Drug Delivery**; PhD Thesis, University of Nottingham.
- Daly, M.M. and Knorr, D. **Biotechnology Progress** 1988; 4 : 76-81.
- Daugherty, A.L., Liggitt, H.D., McCabe, J.G., Moore, J.A. and Patton, J.S. **Int. J. Pharm.** 1988; 45 : 197-206.
- Davis, B.D. **J. Clin Invest.** 1943; 22: 753.
- Dima, F., Vasilescu, M., Popovici, I., Dorneanu, V., Mindreici, I and Gherasim, P. **Rev. Med.-Chir.** 1987; 91 : 533-536.
- Dinsdale, A. and Moore, F. (eds.) 1962; **Viscosity and its Measurement**, Chapman and Hall Limited, London.
- Doig, P., Smith, N.R., Todd, T. and Irvin, R.T. **Infection and Immunity** 1987; 55 : 1517-1522.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. **Anal. Chem.** 1956; 28 : 350-356.
- E.E.L (Evans Electroselenium Ltd.) 1976; **E.E.L. Nephelometer Head for Turbidity Measurements**, E.E.L., Halstead, Essex.

Einig, H. and Knoll, A.G. **First Advanced Course on Alginates and their Applications**, June 1991, Trondheim, Norway.

Fischer, F.G. and Dörfel, H. **Z. Physiol. Chem.** 1955; 302 :186-203.

Florence, A.T. and Attwood, D. (eds.) 1985; **Physicochemical Principles of Pharmacy**, Macmillan Publishers Ltd., London.

Friend, D.R. and Pangburn, S. **Site Spec. Drug Del.** 1987; 7: 53-106.

Frishman, W. **Am. Heart. J.** 1979; 97 : 663-670.

Gacesa, P. **Carbohydr. Polymers** 1988; 8 : 161-182.

Ganz, A.J. **Food Engineering** June 1974.

Gill, J.F., Deretic, V. and Chakrabarty, A.M. **Microbiol. Sci.** 1987; 4 : 296-299.

Gizurarson, S. **Acta. Pharma. Nord** 1990; 2 : 105-122.

Glicksman, M. 1969; in **Gum Technology in the Food Industry**, Academic Press, London.

Gluskey, F.J. et al. **J. Dairy Sci.** 1969; 52 : 1181.

Gonçalves, M.P., Bourgeois, C.M., Lefebvre, J. and Doublier, J.L. **Sci. Aliments** 1986; 6 : 333-347.

Gordon, C.A., Hodges, N.A. and Marriott, C. **Antimicrob. Agents and Chemother.** 1984; 25 : 673-675.

- Gordon, C.A., Hodges, N.A. and Marriott, C. **J. Antimicrob. Chemother.** 1988; 22 : 667-674.
- Gorin, P.A.J. and Spencer, J.F.T. **Can. J. Chem** 1966; 44 : 993-998.
- Govan, J.R.W., Fyfe, J.A.M. and Jarman, T. **J. Gen. Microbiol.** 1981; 125 : 217-220.
- Graham, T., **Phil. Trans. Roy. Soc. London** 1861; 151: 183.
- Graham, H.D. and Thomas, L.B. **J. Pharm. Sci.** 1961; 50 : 483-486.
- Graham, H.D. and Thomas, L.B. **J. Pharm. Sci.** 1962; 51 : 988-992.
- Graham, H.D., Baker, Y.M. and Njoku-obi, A.N. **J. Pharm. Sci.** 1963; 52 : 192-198.
- Grant, G.J., Morris, E.R., Rees, D.A., Smith, P.J.C. and Thom, D. **FEBS Letters** 1973; 32(1) : 195-198.
- Grasdalen, H., Larsen, B. and Smidsrød, O. **Carbohydr. Res.** 1977; 56 : C11-C15.
- Grasdalen, H., Larsen, B. and Smidsrød, O. **Carbohydr. Res.** 1979; 68 : 23-31.
- Grasdalen, H., Larsen, B. and Smidsrød, O. **Carbohydr. Res.** 1981; 89 : 179-191.
- Grasdalen, H. **Carbohydr. Res.** 1983; 118: 255-260.
- Grasdalen, H. and Smidsrod, O. **Carbohydr. Polymers** 1987; 7 : 371-393.

- Green, A.A. **J. Am. Chem. Soc.** 1933; 55 : 2331-2336.
- Guiseley, K.B. et al. 1980; in **Handbook of Water Soluble Gums and Resins**, ed. Davidson R.L.; McGraw-Hill, London.
- Ha, Y.W., Thomas, L.A., Dyck, L.A. and Kunkel, M.E. **J. Food Sci.** 1989; 54 : 1336-1340.
- Habib, F.S., Ismail, S., El-Shanawany, S. and Fouad, E.A. **Eur. J. Pharm. Biopharm.** 1991; 37 : 38-41.
- Harding, S.E. and Johnson, P. **Biochem. J.** 1985; 231 : 543-547.
- Harding, S.E., 1988; in **Gums and Stabilisers for the Food Industry 4**, eds. Phillips, G.O., Wedlock, D.J. and Williams, P.A. IRL Press, Oxford.
- Harrison, G.E., Carr, T.E.F., Sutton, A. and Humphreys, E.R. **Nature (London)** 1969; 224 : 1115-1116.
- Harmuth-Hoene, A. and Schlenz, R. **J. Nutr.** 1980; 110 : 1774.
- Haug, A. **Acta Chem. Scand.** 1961; 15 : 1794-1795.
- Haug, A. and Larsen, B. **Acta Chem. Scand.** 1962; 16 : 1908-1918.
- Haug, A., Larsen, B. and Smidsrød, O. **Acta Chem. Scand.** 1966; 20 (1) : 183-190.
- Haug, A., Larsen, B. and Smidsrød, O. **Acta Chem. Scand.** 1967a; 21: 691-704.
- Haug, A., Myklestad, S., Larsen, B. and Smidsrød, O. **Acta Chem. Scand.** 1967b; 21: 768-778.

- Haug, A. and Smidsrød, O. **Acta Chem. Scand.** 1970; 24 : 843-854.
- Haug, A., Larsen, B. and Smidsrød, O. **Carbohydr. Res.** 1974; 32 : 217-225.
- Hayakawa, K., Santerre, J.P. and Kwak, J.C.T. **Macromolecules** 1983; 16 : 1642-1645.
- Hill, R.D. and Zadow, J.G. **J. Dairy Res.** 1978; 45 : 77.
- Hirai, S., Yashiki, T., Matsuzawa, T. and Mima, H. **Int. J. Pharm.** 1981; 7 : 317-325.
- Hughes, L. et al. **J. Text. Studies** 1980; 11 : 247.
- Hussain, A., Hirai, S. and Bawarshi, R. **J. Pharm. Sci.** 1979; 68 : 1196.
- Hussain, A., Hirai, S. and Bawarshi, R. **J. Pharm. Sci.** 1980a; 69 : 1240.
- Hussain, A., Hirai, S. and Bawarshi, R. **J. Pharm. Sci.** 1980b; 69 : 1411-1413.
- Imeson, A.P., Ledward, D.A. and Mitchell, J.R. **J. Sci. Food Agric.** 1977; 28 : 661.
- Imeson, A.P., Watson, P.R., Mitchell, J.R. and Ledward, D.A. **J. Food Technology** 1978; 13 : 329-338.
- Inagaki, M., Sakakura, Y., Itoh, H., Ukai, K. and Miyoshi, Y. **Rhinology** 1985; 23 : 213-221.
- Kankura, T., Kurashina, S. and Nakao, M. **J. Lab. Clin. Med.** 1974; 83 : 840-844.

Kawashima, S., Nishiura, N., Noguchi, T. and Fujiwara, H. **Chem. Pharm. Bull.** 1989; 37 : 766-770.

Kawashima, S., Sugimura, N., Noguchi, T. and Fujiwara, H. **Chem. Pharm. Bull.** 1990; 38 : 498-505.

Keipert, S., Becker, J., Schultz, H.H. and Voigt, R. **Die Pharmazie** 1973; 28(3) :145-183.

Keith, A.D. 1990; **United States Patent** 4980150.

Keleman, G. **Arch. Otolaryngol.** 1947; 45 : 159-168.

Kemp, W., 1975; in **Organic Spectroscopy**, Macmillan Ltd., London.

Klotz, I.M. **J. Amer. Chem. Soc.** 1946; 68 : 2299-2304.

Kohn, R. **Pure Applied Chem.** 1975; 42 : 371-397.

Kohn, R. **Carbohydr. Res.** 1987; 160 : 343.

Kostial, K. et al. **Environ. Res.** 1971; 4 : 360-363.

Larsen, B., Smidsrød, O., Painter, T. and Haug, A. **Acta Chem. Scand.** 1970; 24(2) : 726-728.

Launay et al. 1986; in **Functional Properties of Food Macromolecules**, eds. Mitchell, J.R. and Ledward, D.A., Elsevier, London.

Ledward, D.A. 1979; in **Polysaccharides in Food**, eds. Blanshard J.M.V. and Mitchell, J.R., Butterworths, London.

- Lee, V.H.L., Yamamoto, A. and Kompella, V.B. **Crit. Rev. in Ther. Drug. Carr. Sys.** 1991; 8 : 91-192.
- Linker, A. and Jones, R.S. **Nature** (London) 1964; 204 : 187-188.
- Linker, A. and Jones, R.S. **J. Biol. Chem.** 1966; 241(6) : 3845-3851.
- Mackie, W. **Carbohydr. Res.** 1971; 20 : 413-415.
- Manzini, G., Césaro, A., Delben, F., Paoletti, S. and Reisenhofer, E. **Bioelectrochem. Bioenerg.** 1984; 12 : 443-454.
- Marshall, J.J. **J Chromatogr.** 1970; 53 : 379-380.
- McArthur, H.A.I. and Ceri, H. **Infection and Immunity** 1983; 42 : 574-578.
- McDowell, R.H. **Rev. Pure Appl. Chem.** 1960; 10 :1-19.
- McDowell, R.H. **J. Soc. Cosmet. Chem.** 1970; 21 : 441-457.
- McDowell, R.H. 1977; in **Properties of Alginates**, Alginate Industries Limited, London.
- McKay, J.E., Stainsby, G. and Wilson, E.L. **Carbohydr. Polymers** 1985; 5: 223-236.
- McMartin, C., Hutchinson, L.E.F., Hyde, R. and Peters, G.E. **J. Pharm. Sci.** 1987; 76 : 535-540.
- McNeely, W.H. and Kovacs, P., 1975; Chapter 17 in **The Physiological Effects of Food Carbohydrates** eds. A Jeanes and J. Hodge, A.C.S. Symposium Series No 15.

- Melia, C.D. **Crit. Rev. in Ther. Drug. Carr. Sys.** 1991; 8 : 395-421.
- Mitchell, J.R. and Blanshard, J.M.V. **J Text. Studies** 1976; 7 : 219-234.
- Mitchell, J.R. 1979; in **Polysaccharides in Food**, eds. Blanshard, J.M.V. and Mitchell, J.R., Butterworths, London.
- Mohamed, S.B. and Stainsby, G. **Food Chem.** 1984; 13 : 241-255.
- Mohamed, S.B. and Stainsby, G. **Food Chem.** 1985; 18 : 193-197.
- Moore, W. and Elder, R.L. **Nature** (London) 1965; 206 : 841-842.
- Morley, S.D., Abraham, R.J., Haworth, I.S., Jackson, D.E., Saunders, M.R. and Vinter, J.G. **J. Comput. Aided Mol. Design** 1991; 5: 475-504.
- Morris, E.R., Rees, D.A., Sanderson, G.R. and Thom, D. **J. Chem. Soc. Perkins Trans.** 1975 :1418-1425.
- Mygind, N., 1985; in **Aerosols in Medicine. Principles, Diagnosis and Therapy** ed. Moren, F., Elsevier, London.
- Negus, V.E. (ed.) **Comparative Anatomy and Physiology of the Nose and Paranasal Sinuses**, 1958; E. and S. Livingstone Ltd., London.
- Nelson, W.L. and Cretcher, L.H. **J. A. Chem. Soc.** 1930; 52: 2130.
- Nichols, W.W., Dorrington, S.M., Slack, M.P. and Walmsley, H.L. **Antimicrob. Agents and Chemother.** 1988; 32: 518-523.
- Oakenfull, D. 1990; in **Gums and Stabilisers for the Food Industry** **5**, IRL Press, Oxford.

- Okazaki, M., Furuya, K. Tsukayama, K. and Nisizawa, K. **Bot. Marina** 1982 ; 25 :123-131.
- Painter, T., Smidsrød, O., Larsen, B. and Haug, A. **Acta Chem. Scand.** 1968; 22(5) :1637-1648.
- Paoletti, S., Murano, E. and Skjåk-Bræk, G. **Proc. Workshop on Phycocolloids and Fine Chemicals** Sept. 1987, Trieste, Italy.
- Paoletti, S. Personal communication, 1989.
- Parr, G.D. **Pharm. Int.** 1983; 4 : 202-205.
- Patrick, G. **Nature** (London) 1967; 216 : 815-816.
- Penman, A. and Sanderson, G.R. **Carbohydr. Res.** 1972; 25 : 273-282.
- Percival, E. 1970; in **The Carbohydrates: Chemistry and Biochemistry**, 2nd edn., eds. Pigman, W. and Horton, D., Academic Press, London and New York.
- Pfister, G., Bahadir, M. and Korte, F. **J. Controlled Release** 1986; 3 : 229-233.
- Popp, J.A. and Martin, J.T. **Amer. J. Anat.** 1984; 169 : 425-436.
- Porath, J. and Flodin, P. **Nature** 1959; 183 : 1657-1659.
- Price, N.C. and Dwek, R.A., 1979; in **Principles and Problems in Physical Chemistry for Biochemists** 2nd Edn., Oxford Science Publications, Oxford.
- Proctor, D.F. **Am. Rev. Resp. Dis.** 1977; 97-125.

- Rees, D.A. **Biochem. J.** 1972; 126 : 257-273.
- Reisenhofer, E., Césaro, A., Delben, F., Manzini, G. and Paoletti, S. **Bioelectrochem. Bioenerg.** 1984; 12 : 455-465.
- Rose, H.E. and Quarterman, J. **Environ. Res.** 1987; 42 : 166-175.
- Schlemmer, V. **Food Chem.** 1989; 32 : 223-234.
- Seale, R., Morris, E.R. and Rees, D.A. **Carbohydr. Res.** 1982; 110 : 101-112.
- Seely, G.R. and Hart, R.L. **Biopolymers** 1979; 18 : 2745-2768.
- Seely, G.R. and Knotts, R.R. **Carbohydr. Polymers** 1983; 3 : 109-127.
- Segi, N., Yotsuyanagi, T. and Ikeda, K. **Chem. Pharm. Bull.** 1989; 37 : 3092-3095.
- Shand, D.G., Nuckalls, E.M., and Oates, J.A. **Clin. Pharmacol. Ther.** 1970; 11 : 112-115.
- Sherys, A.Y., Gurov, A.N. and Tolstoguzov, V.B. **Carbohydr. Polym.** 1989; 10 : 87-102.
- Skjåk-Bræk, G., Larsen, B. and Grasdalen, H. **Carbohydr. Res.** 1986; 154 : 239-250.
- Skjåk-Bræk, G., Grasdalen, H. and Smidsrød, O. **Carbohydr. Polymers** 1989; 10 : 31-54.
- Slack, M.P.E. and Nichols, W.W. **The Lancet** (ii) 1981: 502-503.

- Smidsrød , O. and Haug, A. **Acta Chem. Scand.** 1968; 22 :
1989-1997.
- Smidsrød, O. and Whittington, S.G. **Macromolecules** 1969; 2 :
42-44.
- Smidsrød, O., Haug, A. and Lian, B. **Acta Chem. Scand.** 1972a; 26 :
71-78.
- Smidsrød, O. and Haug, A. **Acta Chem. Scand.** 1972b; 26 : 79-88.
- Smidsrød, O., Glover, R.M. and Whittington, S.G. **Carbohyd. Res.**
1973; 27 : 107.
- Smidsrød, O. **Faraday Discuss. Chem. Soc.** 1974; 57 : 263-274.
- Smidsrød, O. 1979; **27th IUPAC Congress** Helsinki, ed. A.
Varmavouori, Pergamon Press, Oxford.
- Stainsby, G. **Food. Chem.** 1980; 6 : 3.
- Stanford, E.C.C. 1881; **British Patent** 142.
- Steinnes, A. **Giordian** 1975; 7-8 : 228-230.
- Stockwell, A.F., Davis, S.S. and Walker, S.E. **J. Controlled Release**
1986; 3 : 167-175.
- Takahashi, Y. **J. Inclusion Phenom.** 1987; 5 : 525-534.
- Tangen, O., Berman, H.J. and Marfey, P. **Thromb. Diath.**
Haemorrh. 1971; 25 : 269-278.

- Tannenbaum, C.S., Hastie, A.T., Higgins, M.L., Kueppers, F. and Weinbaum, G. **Antimicrob. Agents and Chemother.** 1984; 25 : 673-675.
- Thom, D., Dea, I.C.M., Morris, E.R. and Powell, D.A. **Prog. Fd. Nutr. Sci.** 1982; 6 : 97-108.
- Toft, K. **Prog. Fd . Nutr. Sci.** 1982; 6 : 89-96.
- Tucker, I.J., Gulshan, H.A. and Stewart, P.J. **Aust. J. Hosp. Pharm.** 1988; 18 :196-199.
- Van de Donk, H.J.M. and Merkus, F.W.H.M. **J. Pharm. Sci.** 1982; 71 : 595-596.
- Verhoef, J. et al. **Proceed. Intern. Symp. Control. Rel. Bioact. Mater.** 1989; 16 : 85-86.
- Vinter, J.G., Davis, A. and Saunders, M.R. **J. Comput. Aided Mol. Design** 1987; 1: 31-51.
- Washington, N., Washington, C., Wilson, C.G. and Davis, S.S. **Int. J. Pharm.** 1986; 34 : 105-109.
- Washington, C. (ed.) 1992 **Particle Size Analysis in Pharmaceutics and Other Industries**, Ellis Horwood Limited, Chichester, West Sussex.
- Weder, H.G., Schildknecht, J. and Kesselring, P. **Am. Lab.** 1971; 3 : 15.
- Wingender, J., Volz, S. and Winkler, V.K. **Appl. Microbiol. Biotechnol.** 1987; 27 : 139-145.

Winter, G.D. **Nature, London** 1962; 193 : 293-294.

Wölbling, R.H., Becker, G. and Forth, W. **Digestion** 1980; 20 :
403-409.

Zipper, J., Wheeler, R.G., Potts, D.M. and Rivera, M. **Br. Med. J.**
1983; 287 : 1245-1246.