CHEMISTRY AND IMMUNOCHEMISTRY OF CARRAGEENANS

By

VINCENT LUIGI DI NINNO, B.Sc.

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

October (1978)

CHEMISTRY AND IMMUNOCHEMISTRY OF CARRAGEENANS

DOCTOR OF PHILOSOPHY (1978)
(Biology)

McMaster University
Hamilton, Ontario

TITLE: Chemistry and Immunochemistry of Carrageenans

AUTHOR: Vincent Luigi Di Ninno, B.Sc. (McGill University)

SUPERVISOR: Professor E.L. McCandless

NUMBER OF PAGES: xiv; 102.

ABSTRACT

A turbidimetric assay was adapted so as to enable kinetic measurements (rates of precipitation) of antigen-antibody reactions (i.e. agarose medium turbidimetric assay). This technique provides a simple rapid method for investigating differences in the immunochemical reactivity of carrageenans of a given type to a specific antibody preparation. Antibodies to κ- λ- and ι-carrageenan were raised in goats and characterized; the antigenic structural components responsible for interaction with the antibody were determined. An investigation of the finer details of carrageenan structural, chemical and immunochemical variability was undertaken. The agarose medium turbidimetric assa was used as an efficient probe to detect gross or subtle variations in carrageenan structure. These structural variations were further characterized by chemical and physical procedures. A carrageenan from *P. middendorfii (P. franciscana)* was found to represent a unique polymer herein referred to as π-carrageenan.

ACKNOWLEDGMENTS

The author would like to express sincere gratitude to Drs. L. Prevec and J. Gauldie for their supervision during the course of this study and to Dr. E.L. McCandless for her interest and expert guidance.

The author thanks Drs. J.M Rosenfeld and M. Hatton for assistance in the use of gas-liquid chromatography and mass spectroscopy and to Dr. R.A. Bell for the helpful discussions on the interpretation of the mass spectra and for his assistance in N.M.R. spectroscopy.

The author expresses appreciation to Drs. J.S. Craigie, L. Chen, C.J. Dawes, J.E. Hansen, J. Stein, F. Loewus, A. Nonamura, M. Doty, J. Cabioch, Marine Colloids Inc. and Litex Ltd. for supplying algal material and to C. Vollmer for extraction of the carrageenans.

The author thanks Nancy Lyons and Kathy Howard for assistance with the typing of the thesis.

TABLE OF CONTENTS

CHAPTER III

CHEMISTRY AND IMMUNOCHEMISTRY

OF CARRAGEENANS FROM

EUCHEUMA AND RELATED ALGAL SPECIES

CHAPTER IV

IMMUNOCHEMISTRY OF KAPPA-TYPE

CARRAGEENANS FROM CERTAIN

MARINE RED ALGAE

CHAPTER V

IMMUNOCHEMISTRY OF LAMBDA-TYPE

CARRAGEENANS FROM CERTAIN

MARINE RED ALGAE

LIST OF FIGURES

LIST OF TABLES

THESIS RELATED

PUBLICATIONS

1. V. DiNinno & E.L. McCandless. "Agarose Medium Turbidimetric Assay for Cross-Reacting Antigens." J. Immunol. Meth. 1977. 17, 73-79.

2. V. DiNinno & E.L. McCandless. "Anti-Carrageenans." Immunochemistry. 1978. 15, 273-274

3. V. DiNinno & E.L. McCandless. "The Immunochemistry of λ-type Carrageenans from Certain Red Algae." Carbohydrate Research. 1978 (In Press).

4. E.L. Mcandless, M.J. Evelegh, C.M. Vollmer and V.L. DiNinno. "Immunological Characterization of 'Lambda' Carrageenans. International Seaweed Symposium V. 9, Santa Barbara, California. 1978. (In Press).

5. V. DiNinno & E.L. McCandless. "The Chemistry and Immunochemistry of Carrageenans from *Eucheuma* and Related Algal Species." Carbohydrate Research. 1978. (In Press).

6. V.L. DiNinno, E.L. McCandless & R.A. Bell. "Pyruvated Carrageenan from a Marine Red Alga (*Petrocelis* species)." Carbohydrate Research. 1978. (In Press).

7. V.L. DiNinno & E.L. McCandless. "The Immunochemistry of κ-type carrageenans from Certain Red Algae." Carbohydrate Research. 1978. (In Press).

8. V.L. DiNinno, R.A. Bell & E.L. McCandless. "Identification of 4,6-O-(1-carboxyethylidene-D-Galactose by Combined GC/MS." BMS. (accepted for publication).

DEDICATED TO

MY WIFE

TERESA

MY DAUGHTER

ALEXANDRA

AND

MY PARENTS

ELISA AND GIUSEPPEANTONIO

DI NINNO

INTRODUCTION

The sugars which comprise the polysaccharides of cell walls of marine algae are characteristic of particular algal groups. The marine red algae produce sulphated polygalactans, which include the carrageenans. These are widely used in the food industry and are known to possess a multitude of biological and immunological properties[1].

Historically, carrageenans were fractionated into two main components on the basis of their solubility in the presence of potassium ions[2]. The insoluble fraction was termed kappa (κ) carrageenan and the soluble fraction lambda (λ) carrageenan. Since then other carrageenans have been described: mu (μ) carrageenan[3], iota (ι) carrageenan[4], xi (ξ) carrageenan[5], nu (ν) carrageenan[6], and pi (π) carrageenan[7]. Classification can no longer be based on solubility properties alone but must be supplemented by structural and chemical characterization. The idealized, major repeating units of known carrageenans are shown in Fig. 0,1. Carrageenans consist of alternating β (1,4), α (1,3) linked units of D-galactose. The 3-linked units can be galactose, galactose-4-SO_4, galactose-2-SO_4, and 4,6-O-(1-carboxyethylidene)-D-galactose-2-SO_4. The 4-linked units can be galactose-2,6-di-SO_4, galactose-6-SO_4,

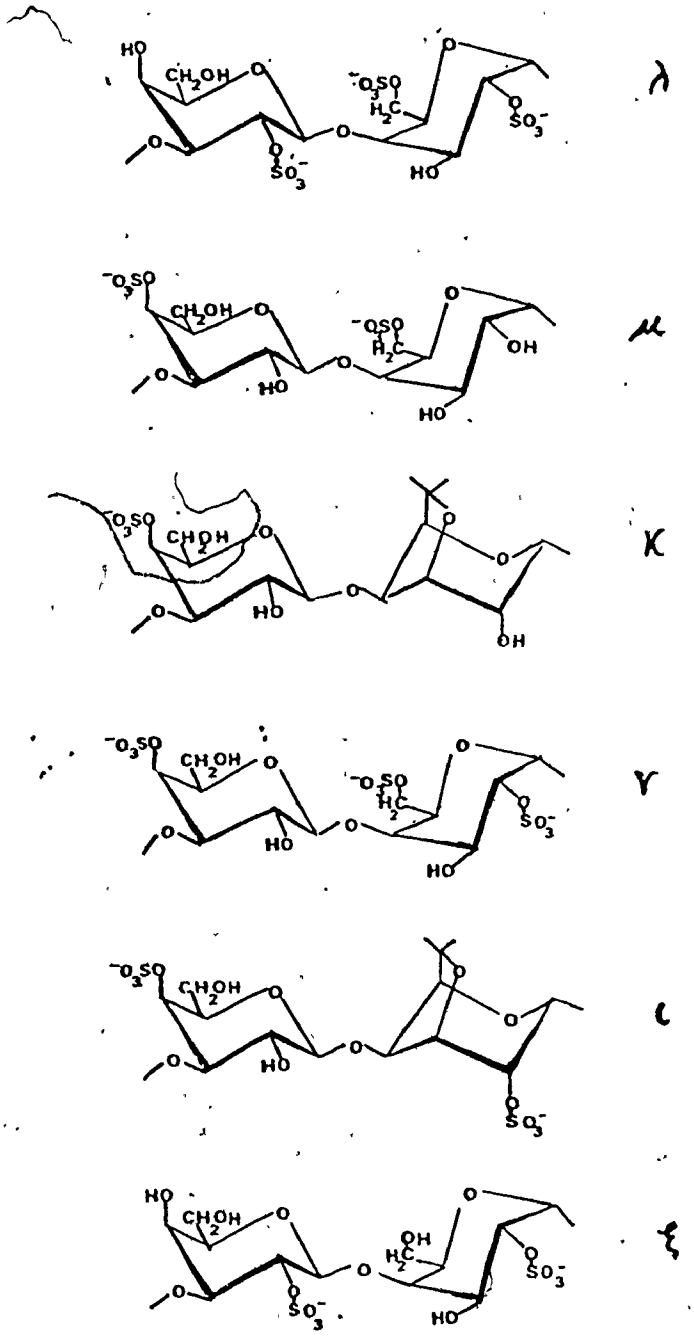

Fig. 0,1: Idealized major repeating unit of various carrageenans.

galactose-2-SO_4, 3,6 anhydrogalactose or 3,6 anhydrogalactose-2-SO_4. The anhydride unit may be derived from galactose-6-SO_4 by alkaline[8] or enzymic[9] elimination of the sulphate on C-6 of the pyranose ring of the 4-linked unit. Such a phenomenon has suggested that μ-carrageenan units are precursors to kappa-carrageenan units and that ν-carrageenan units are precursors to ι-carrageenan units.

For purposes of discussion and orientation, we will subdivide the carrageenans into two major classes (Table 0,1), the kappa-carrageenan class consisting of those carrageenan types containing the 3,6 anhydride as one of its major components, and the lambda-carrageenan class consisting of those carrageenan types which lack this structural component. In other words the 4-linked 3,6

Table 0,1. Classification of Carrageenans

Lambda-Carrageenan Class	Kappa-Carrageenan Class
λ-carrageenan	κ-carrageenan
ξ-carrageenan	ι-carrageenan
π-carrageenan	furcellaran
ν-carrageenan	
μ-carrageenan	

anhydride will serve as the reference point for class

assignment. Within each class several carrageenan types have been described and these are defined by their ester-SO_4 content and position as well as their solubility properties in KCl. Within each type of sulphated polygalactan, deviations and variations from the idealized structure may occur. The major repeating units for each carrageenan, indicated in Fig. 0,1., may be interrupted or masked by the occurrence of other residues. In the kappa-class, for example, the repeating disaccharide unit may be interrupted by β (1-4) linked galactose-2,6-di-SO_4, galactose-6-SO_4 as well as by non-sulphated residues. These structures have been termed "kinking" residues because of their effects on the molecular configuration of the polymer[10]. The idealized repeating structure, therefore, may account for as little as 60% of the total carrageenan preparation. This type of variation has led to a discussion of hybrid molecules[5], although it remains difficult in the lambda-class polymers to ascertain whether the carrageenan molecules are true hybrids or simply mixtures of different polymers[6]. In the kappa-class carrageenans, if the content of kinking residues is sufficiently high the carrageenan becomes soluble in KCl. These molecules then do represent true hybrids[5].

The different properties displayed by the two classes of carrageenans have been attributed to the 3,6 anhydride.

This residue is responsible for the secondary, tertiary and quaternary structures observed in the kappa-class and which is so essential to the gelling properties of these carrageenan types[11].

While differences between κ and λ type carrageenans are clearly defined, the chemical and physical differences observed within a given type of carrageenan are more difficult to interpret. One observes good consistency with the chemical data of carrageenans extracted from one algal species but slight differences might be seen in the infrared spectrum or chemical content of one of the constituents of the carrageenan extracted from another algal species producing the same carrageenan type. The question arises then as to the relationship between the two carrageenans; that is to say, are the differences significant and how do they translate to a structural basis? In order to investigate these questions, a probe is needed which is sensitive to subtle variations in primary, secondary, and tertiary structure displayed by these polysaccharides. The demonstration that carrageenans are immunogenic[12] offers an ideal tool. Indeed antigen-antibody (Ag-Ab) interaction is quite susceptible to minor alterations in the structure of a particular antigen.

A large volume of often contradictory reports are accumulating on the effects of carrageenans on the immune

system[13,14,15]. The confusion in such literature might, in part, be due to the use of carrageenans that are not clearly defined as to source, type, chemical and structural properties. A better understanding of carrageenan structure and clearly defined methods of characterization might shed some light on, or at least produce some uniformity in the effects of various carrageenans on immunobiological phenomena.

This thesis concerns itself with the investigation of chemical and immunochemical variations within given types of carrageenans. The author has undertaken an investigation of the finer details of carrageenan structural, chemical and immunochemical variability. The significance of gross or subtle variations in carrageenan structure, detected immunochemically and further characterized chemically and physically, to the taxonomy of the algae will be discussed in those chapters where the differences bear obvious taxonomic importance.

CHAPTER I

AGAROSE MEDIUM TURBIDIMETRIC ASSAY FOR CROSS-REACTING ANTIGENS

INTRODUCTION

Several methods are available for the investigation of cross-reactive antigens; the most widely used are the Ouchterlony double diffusion technique and the semi-quantitative antibody precipitin and agglutinin reactions. These methods are tedious, time consuming and often fail to provide quantitative information as to the structural relationship between cross-reacting antigens.

If we place an anti-κ-carrageenan or an anti-λ-carrageenan in the center well of an immunodiffusion plate and react these to κ- and λ-carrageenan, the specificity of the anti-carrageenans can be clearly seen in fig. I,1. The anti-κ-carrageenan reacts with κ and not λ-carrageenan. The reverse is true for the anti-λ-carrageenan. Kappa and λ-carrageenan, as previously mentioned, represent the two extremes of carrageenan classes. None of the constituent residues are identical in these two carrageenan types. If, however, we react the very same anti-carrageenan preparations to carrageenan types of the same class, reactions of identity

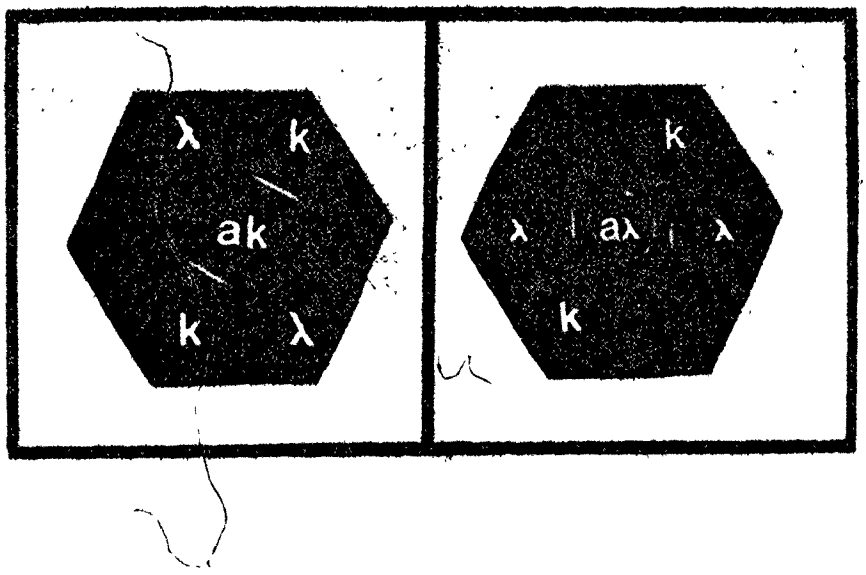

Fig. I, 1: Reactivity of anti-κ-carrageenan (aκ) and anti-λ-carrageenan (aλ) to κ- and λ-carrageenan from *C. crispus*.

predominate (Fig. I,2). Such a technique is not satisfactory for detailed immunochemical characterization of carrageenans. Reactions of identity on immunodiffusion plates may arise by one of the following processes; a) the occurrence of different antigenic determinants on the antigens reacting with the same antibody but with different affinity or b) the occurrence of identical determinants on the antigens. It is thus possible to miss both qualitative and quantitative differences among cross-reacting antigens. It may be more informative, therefore, to utilize a technique into which both the quantity of determinants per weight of antigen and antigen-antibody affinity have an input into the ultimate analysis and quantitation of Ag-Ab interaction.

In this chapter we will describe a simple, rapid, method for assaying rates of precipitation based on the phenomenon of turbidity. This procedure is then adapted to the investigation and quantitation of cross-reacting carrageenan antigens.

Basis of Agarose Medium Turbidimetric Assay:

Serological precipitation is believed to occur in two phases; a rapid primary phase which involved the combination of antigen and antibody, followed by further interaction in which these initial molecular complexes aggregate and precipitate[16]. This secondary phase has long

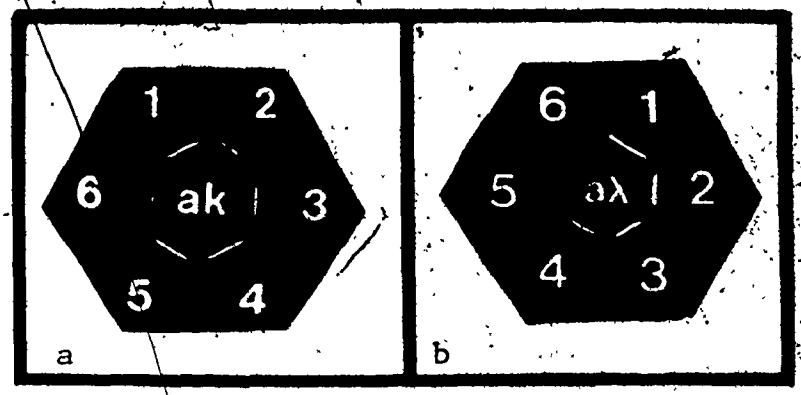

Fig. I,2a: Reactivity of anti-κ-carrageenan to various κ-type carrageenans.
centre well: anti-κ-carrageenan
peripheral wells: 1) and 5) *C. crispus* female insoluble; 2) *G. aghardhii* female insoluble; 3) and 6) *G. aghardhii* female soluble and 4) *C. crispus* female soluble, carrageenans.

Fig. I,2b: Reactivity of anti-λ-carrageenan to various λ-type carrageenans.
centre well: anti-λ-carrag[illegible]
peripheral wells: 1) *C. cri[illegible]* tetrasporic soluble; 2) *P. francoisoana* tetrasporic soluble; 3) *G. corymbifera* tetrasporic soluble; 4) *C. crispus* female soluble; 5) *G. aghardhii* female soluble and 6) *G. papillata* female soluble, carrageenans

been known to result in the development of opalescence[17]. Pope and Healy[18] showed that the rate at which opacity develops in the reaction between diphtheria toxin and equine antitoxin is maximal with equivalent amounts of toxin and antitoxin. Martin[19] found that the turbidity produced by an antigen-antibody precipitate, five minutes after mixing of the components, was directly proportional to the antigen concentration. Boyden *et al.*[20] showed the turbidity measured spectrophotometrically to be correlated with the nitrogen content of the precipitates formed in the interaction of pneumococcal polysaccharides with an anti-pneumococcal polysaccharide antibody preparation.

The reaction of antigen with antibody often manifests itself by the appearance of microscopic aggregates which absorb and diffuse light. Since the absorbance of a substance is proportional to its concentration, spectrophotometry can be used to measure a change in concentration which occurs during a chemical or physical alteration. The reactivity of antigen and antibody can thus be quantitated by measuring the amount of light scattered (nephelometry) or the amount of light transmitted (turbidity). The amount of light which an Ag-Ab reaction suspension will transmit depends on the properties of the antigen, antibody and the complex formed by their interaction. Reproducible results are possible only if we can prevent the complexes from

settling.

We have ascertained that 0.1% agarose supplies the mechanical support for holding the antigen-antibody aggregates in suspension throughout the period of reaction and provides a medium for the uniform development of turbidity. Fig. I,3 indicates the enhanced sensitivity of the assay in the presence of agarose. After a certain amount of time the mixture not containing the agarose is no longer optically stable and continuous kinetic monitoring of the precipitation reaction is no longer possible (Fig. I,3). Turbidity measurements are also influenced by the size of the particles as well as their number. The increased viscosity resulting from the addition of agarose may prevent the Ag-Ab aggregates from clumping and allow the formation of a finer and more uniform precipitate, thus giving an enhanced sensitivity.

Dependence of the Rate of Turbidity Production on Antigen Concentration

The rate of development of turbidity when increasing amounts of κ-carrageenan were reacted with a constant amount of anti-κ-carrageenan antibody can be seen in Fig. I,4. While it is known that the amount of antibody precipitated at equilibrium is dependent on the concentration of both antigen and antibody[16], kinetic studies using the agarose

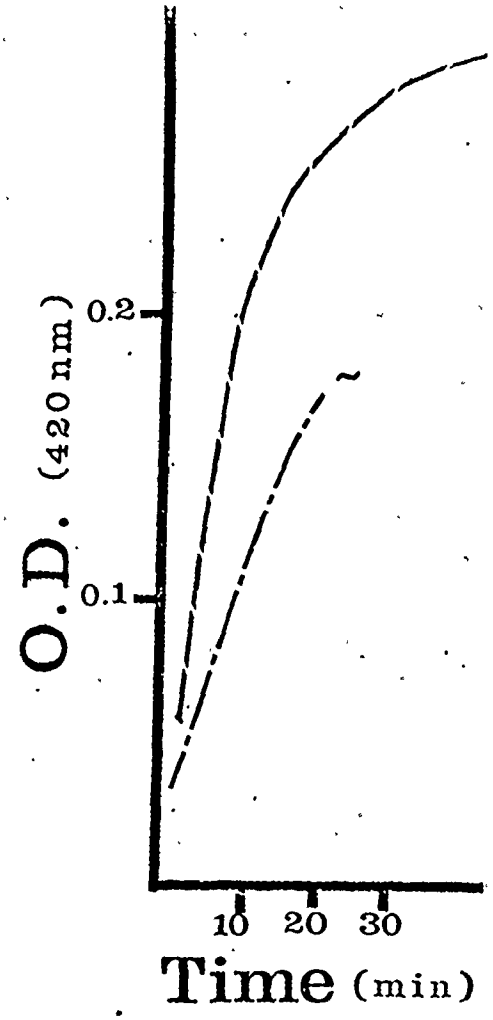

Fig. I,3: Precipitation of anti-κ-carrageenan antibody (G9-γ-676) by κ-carrageenan in a 0.1% agarose medium (— — —) and in the absence of agarose (-·-·-·-·-); as measured by turbidimetric analysis at 420 nm.

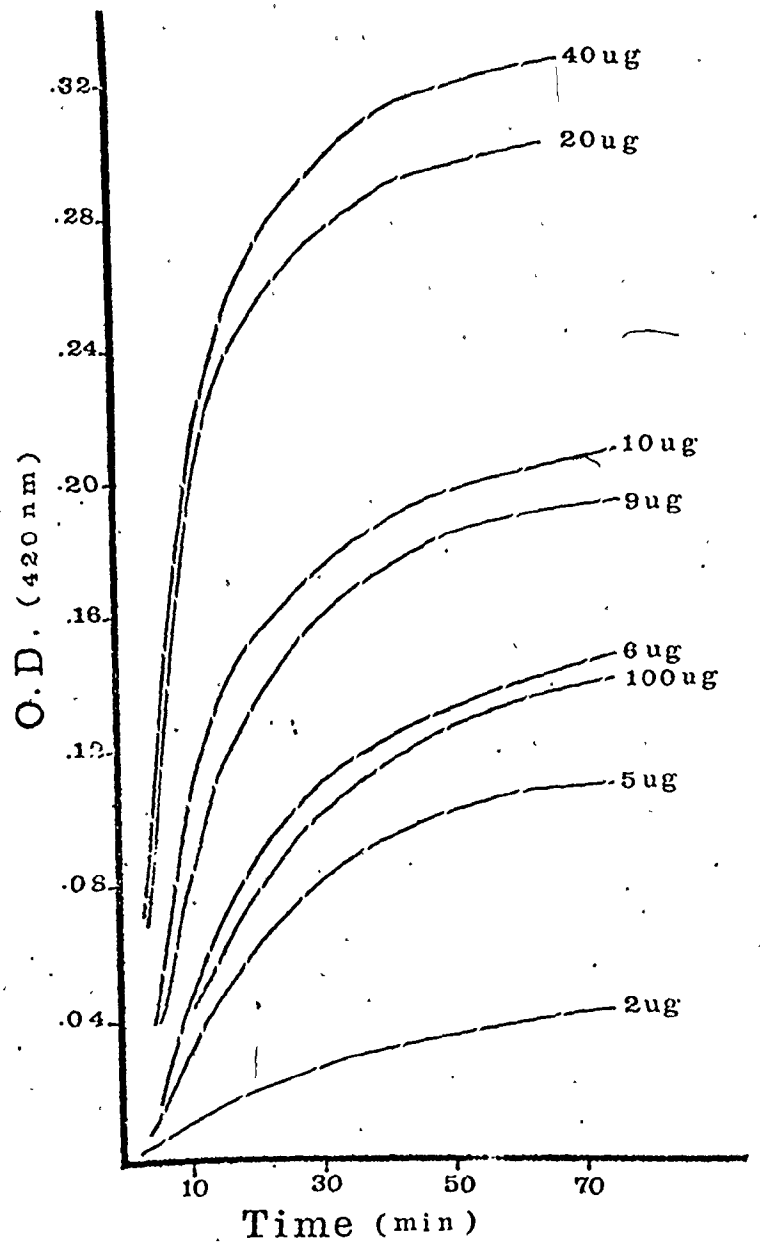

Fig. I,4: Precipitation of anti-κ-carrageenan antibody (G9-γ-676) by varying amounts of κ-carrageenan, as measured by turbidimetric analysis at 420 nm.

medium turbidimetric assay shows that the initial rate of precipitation shows a similar dependence. Plots of initial rate of precipitation vs. antigen concentration (Fig. I,5a) and of equilibrium optical density vs. antigen concentration (Fig. I,5b) resembled precipitin curves obtained when total precipitated Ab nitrogen was measured[21]. The rate of precipitation increased linearly with Ag concentration in the antibody excess zone (Fig. I,5a). The mixture with the greatest rate of turbidity development was considered to contain the reactants in optimal concentration.

Cross-Reacting Antigens

For the analysis of cross reacting antigens, equal amounts (by weight) of homologous antigen and cross-reacting substances were reacted with a constant amount of antibody in a total volume of 3 ml. Both the rates of turbidity production and final equilibrium turvidity were recorded.

The ability of equal amounts of various carrageenans to precipitate anti-κ-carrageenan can be seen in Fig. I,6. The amount of antigen to be used was determined from the plot of initial rate of precipitation vs. antigen concentration (Fig. I,5a) at a point where the rate of turbidity production is most sensitive to changes in the

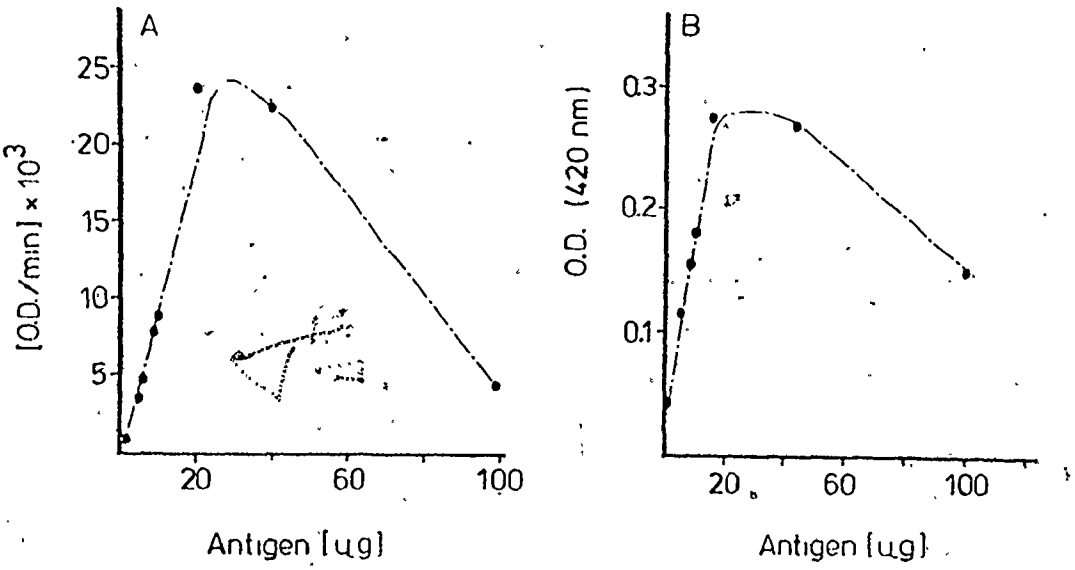

Fig. I,5A: Dependence of the rate of Ag-Ab precipitation (i.e. initial rate of turbidity production) on the amount of antigen in a 3 ml reaction mixture.

Fig. I,5B: Dependence of the total turbidity produced by an Ag-Ab reaction on the amount of antigen in a 3 ml reaction mixture

** The reaction mixture consisted of 0.1% agarose in in 0.01 M. phosphate buffered saline pH 7.5

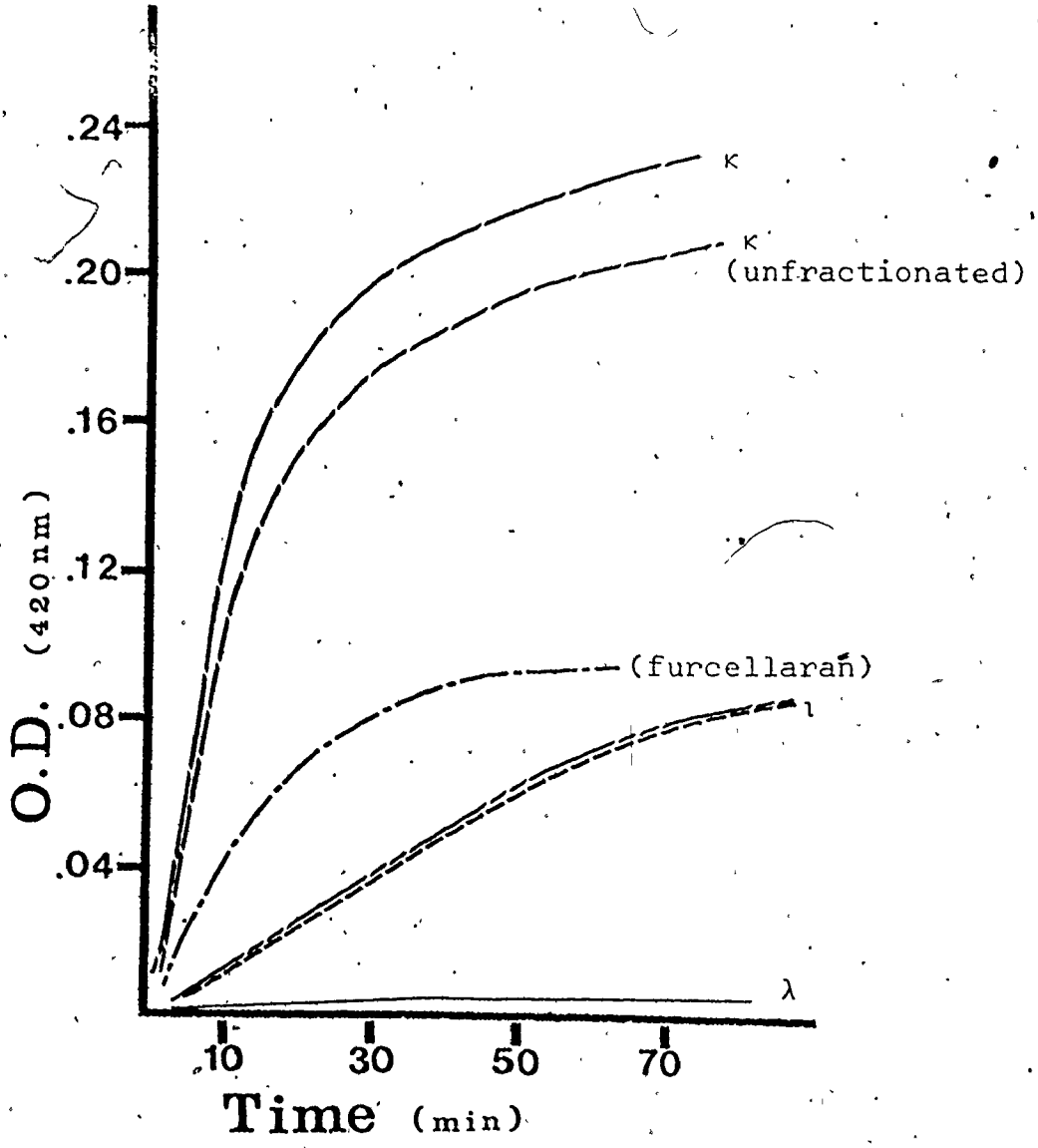

Fig. I,6: Precipitation of a constant amount of anti-κ-carrageenan by equal amounts of *C. crispus*, KCl insoluble (κ-carrageenan) (——— ———); *C. crispus* unfractionated (— — — —), *F. fastigiata* (fucellaran) (— · — · —); *E. spinosum* (— — — —); *E. nudum* (— — — —) and *C. crispus* KCl soluble (λ-carrageenan) (———), carrageenans in a 3 ml reaction mixture (0.1% agarose in 0.01 M PBS pH 7.5) as measured by turbidimetric analysis at 420 nm.

concentration of antigen antibody binding sites (antigenic determinants). Kappa carrageenan, the homologous antigen, was most efficient, followed by furcellaran and iota carrageenans. Lambda carrageenan did not precipitate anti-κ-carrageenan. By keeping the amount of antigen and antibody constant it is possible to quantitate the extent of cross-reactivity and to calculate an index of homology (I.H.) by using the formula:

$$\text{I.H.} = \frac{\text{experimental (precipitation rate X equilibrium O.D.)}}{\text{reference (precipitation rate X equilibrium O.D.)}}$$

where the reference is the homologous antigen. The value for the I.H. will, therefore, range from zero for a non cross-reacting antigen to 1 for the homologous antigen. The index of homology is characteristic of the particular antibody preparation; the specificty of the antibody is always an important parameter in probing cross-reacting antigens. The calculated indices for the various carrageenans used are shown in Table I,1.

DISCUSSION

A serological precipitate is often formed by the interaction of antigen and antibody. The resultant turbidity is, therefore, defined by the properties of the antigen, antibody and the complex formed by their interaction. Given a multivalent antigen and a divalent antibody molecule,

lattice formation and precipitation will occur[16]. The rate of precipitation will depend on the concentration of

Table I,1. Indices of homology to *C. crispus* κ-Carrageenan.

Carrageenan Source & Type	Total Turbidity	Rate of Precipitation OD/min x 10^3	I.H.
Chondrus crispus (♀) KCl insoluble carrageenan (κ-carrageenan)	.225	12.3	1
Chondrus crispus (♀) unfractionated carrageenan	.210	9.6	0.72
Furcellaria fastigiata (Furcellaran carrageenan)	.095	3.9	0.13
Eucheuma nudum (ι-carrageenan)	.080	1.3	0.04
Eucheuma spinosum (ι-carrageenan)	.075	1.3	0.04
Chondrus crispus (λ-carrageenan)	0	0	0

the reactants and on the binding affinity between the antigenic determinant and the antibody combining site(s). For any given antigen the binding affinity in a given antiserum will be constant and by keeping the antibody concentration constant the rate of precipitation should be

dependent only on the concentration of antigenic determinants. In the antibody excess zone the rate of precipitation was found to increase linearly with the antigen concentration until the equivalence point was reached, beyond which addition of more antigen altered the properties of the antigen-antibody molecular complex (Fig. I,5a&b). The rates of production of turbidimetric aggregates for equal amounts of related antigens is dependent on the concentration of antigenic determinants and on the binding affinities of the antibody combining site(s) for these antigenic determinants. The final or equilibrium constitution of the Ag-Ab precipitate is determined principally by the concentration of the reactants[16]. These two parameters (i.e. rate of precipitation and total turbidity) must be considered when quantitating the structural homology of related antigens. Only when the rate of precipitation and total turbidity are identical to that of the homologous antigen can we consider these molecules to be "immunostructurally" identical. A comparison of the index of homology, obtained by the agarose medium turbidimetric assay, for the antigens used show furcellaran to be a better cross-reacting antigen than iota-carrageenan. One can speculate then that κ-carrageenan antigenic determinants occur more frequently in furcellaran than in iota-carrageenan or that furcellaran

has antigenic determinants which more closely resemble kappa-carrageenan. Since at equilibrium both iota-carrageenan and furcellaran display the same total turbidity we favour the latter explanation. This conclusion is also corroborated by chemical analysis[5] which shows the sulphation pattern of kappa-carrageenan to resemble more closely that of furcellaran than iota-carrageenan. The low index of homology of both these carrageenans reflects in part the lower number of determinants per equivalent weight than are found in kappa-carrageenan. Lambda-carrageenan lacks 3,6 anhydro-α-D-galactopyranose as well as galactose-4-SO_4, the two constituent residues of kappa-carrageenan. This is well reflected in the inability to precipitate anti-κ-carrageenan antibody, a total lack of κ-carrageenan-like determinants and an I.H. equal to 0. Unfractionated carrageenan from female algal plants of *C. crispus* also showed a lower index of homology than the homologous, fractionated carrageenan (κ-carrageenan).

The agarose medium turbidimetric assay can be used as a rapid method for evaluating the structural homology between carrageenans which would, otherwise, be difficult to distinguish.

CHAPTER II

NORMAL SERUM-CARRAGEENAN INTERACTION AND ANTI-CARRAGEENANS

INTRODUCTION

The demonstration that carrageenans are immunogenic[12] opens the possibility for rapid immunochemical characterization of carrageenans; but before one can proceed with any immunological characterization one needs to obtain some information as to the specificity of the antibody preparations and the antigenic structural components responsible for Ab-Ab reactivity.

In this chapter, therefore, we evaluate the specificity of various antibody preparations and discuss the carrageenan structural properties which appear to be responsible for their reactivity to the antibody. We will also comment on the interaction of carrageenan with normal serum.

RESULTS

Pre-immune serum from cattle, goats, and humans all show reactivity to κ and λ-carrageenan when tested by the Ouchterlony double technique (Fig. II,1). Lambda carrageenan

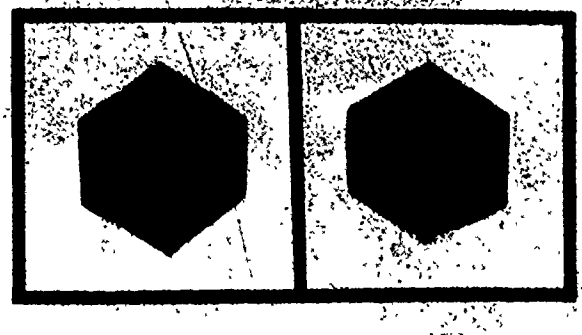

Fig. II,1: Reactivity of carrageenans with normal sera.

A: centre well: λ-carrageenan

1: human normal serum

2: pooled normal goat serum

3: pooled normal cattle serum

B: centre well: κ-carrageenan

1: human normal serum

2: pooled goat serum

3: pooled cattle serum

is more reactive than κ-carrageenan. Neither κ nor λ-carrageenan showed reactivity to human or mouse C-reactive protein when tested by the Ouchterlony double diffusion technique in the presence of Ca^{++} ions.

Pre-immune and immune (G9-anti-κ-carrageenan serum) were fractionated into water insoluble euglobulin (fraction 1) and a 33% saturated ammonium sulphate precipitable water soluble component (i.e. γ G-globulin). Figs. II,2a and 2b show the reactivity of the serum and these fractions to κ and λ-carrageenan. The immune reactivity is seen to be distinct from the normal serum-carrageenan interaction (Figs. II,2a,b). The immune reactivity is found in the γ G-globulin fraction while the pre-immune reactivity is identified with water insoluble euglobulin component(s).

Antisera to various carrageenans were raised in goats and antibody prepared by precipitation of the γ G-globulin fraction (i.e. 33% saturated $(NH_4)_2SO_4$ insoluble fraction) after removal of water insoluble euglobulin. The immunochemical reactivity of κ, λ, ι, and alkaline borohydride (OH^-BH_4) modified λ-carrageenans (Fig. II,3) were assayed by the agarose medium turbidimetric assay and reported in Fig. II,4a-4d.

DISCUSSION

Carrageenan-Normal Serum Interaction

Numerous interactions between protein and macro-

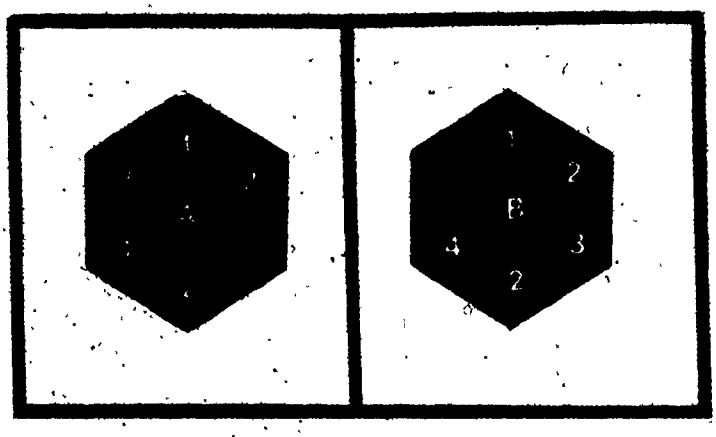

Fig. II,2: Reactivity of carrageenans with normal (A) and immune (B) serum fractions

A: centre well: λ-carrageenan

1: pooled normal goat serum

2: κ-carrageenan

3: water soluble euglobulin from pooled normal goat serum

4: water insoluble euglobulin from pooled normal goat serum

B: centre well: κ-carrageenan

1: G9-anti-κ-carrageenan serum

2: λ-carrageenan

3: G9-γ-477 (33% $[NH_4]_2SO_4$ precipitable water soluble euglobulin), [i.e. IgG]

4: water insoluble euglobulin from G9-anti-κ-carrageenan

molecular polyanions (electrolytes) such as deoxyribonucleic acid, heparin, chondroitin sulphate, dextran sulphate, hyaluronic acid, and synthetic high molecular weight polymers, have been reported and these have been extensively reviewed by Klotz[22]. Protein-polyanion interactions are generally observed at a pH below the isoelectric point of the protein; more akin to physiological phenomena are those interactions which occur between normal serum components with various non-protein substances at physiological pH. Little is known concerning the significance and mechanisms of the latter interactions.

Bernfeld *et al.*[23] have demonstrated that, at physiological pH, sulphated amylopectin is a specific precipitant for beta-lipoprotein. This specific interaction was later adapted to the isolation, chemical characterization and quantitative turbidimetric determination of beta-lipoprotein.

Amyloid P-component (APC) occurs in normal serum and has been shown to bind to agarose columns[24]. It has a molecular weight (MW) of 233,000 and a pentagonal structure as demonstrated by electron microscopy[25]. It has an amino acid sequence similar to C-reactive protein and may itself be an acute phase protein.

In the course of preparing antisera to carrageenans I have observed that at physiological pH carrageenan

precipitates a serum component which like APC is a water insoluble euglobulin. This carrageenan precipitable component (CPC) is not C-reactive protein. It is also clear from Fig. II,2b that the interaction between carrageenan and normal serum component(s) is distinct from the immune globulin fraction induced by immunization.

Anti-carrageenan-Carrageenan Reactions

The ideal structures of the various carrageenans used in this chapter are shown in Fig. II,3. The reactivities of these carrageenans with anti-carrageenan preparations are indicated in Figs. II,4a-4d. The reactivity of the anti-λ-carrageenan preparation (Fig. II,4a) is specific for λ-carrageenan. Conversion of the 6-SO_4 to the 3,6 anhydride by OH^-BH_4 treatment eliminates the reactivity of the polymer to the anti-λ-carrageenan. It is suggested that some structural feature associated with gal-6-SO_4 is responsible for Ag-Ab reactivity. This further supported by the observation that λ-type carrageenans which lack 6-SO_4 (i.e. ξ-carrageenan) do not react with this anti-λ-carrageenan preparation; these data will be presented in chapter V.

The specificity of the anti-κ-carrageenan preparations (Figs. II,4b,4c) is indicated by the lack of reactivity to λ-carrageenan and decreased reactivity to

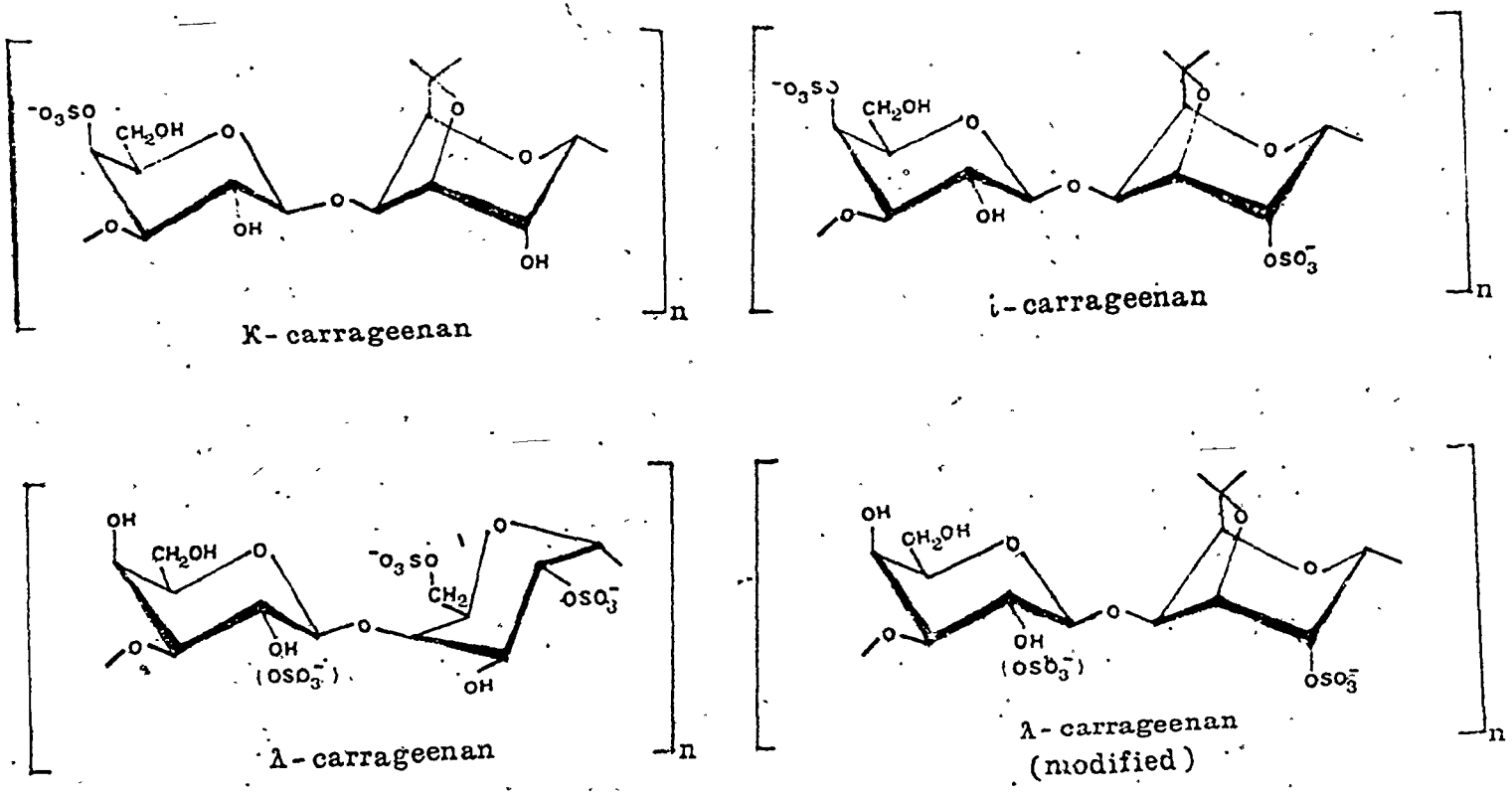

Fig. II,3: The idealized structural formulas of the various carrageenan types.

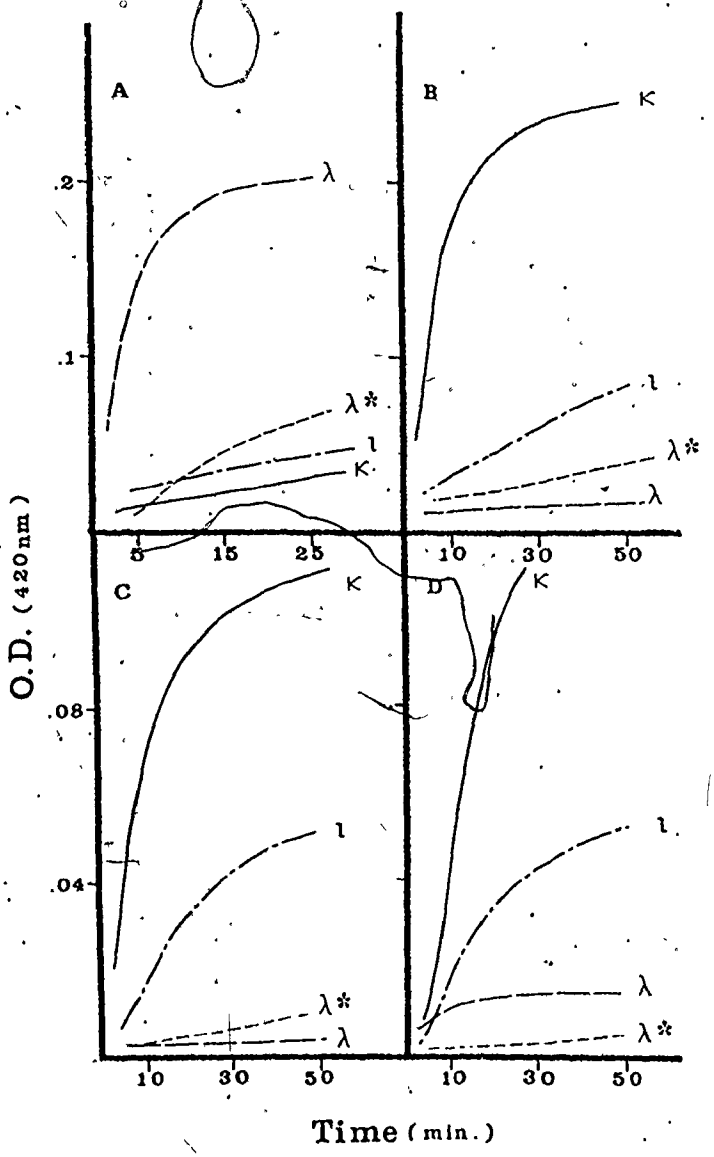

Fig. II, 4A-4D: The reactions of κ- (————), ι- (— — — —), λ-(— — — —) and $OH^- BH_4$ λ-carrageenan (— — — — —) with A) anti-λ-carrageenan G7-γ-477, B) anti-κ-carrageenan G9-γ-477, C) anti-κ-carrageenan G15-γ-777, D) anti-ι-carrageenan G14-γ-1276 as measured by the agarose medium turbidimetric assay.

λ* = alkaline modified λ

ι-carrageenan. It is suggested that the antibody reactivity is directed to a structural feature associated with 3,6 anhydrogalactose (3,6 AG). The importance of the 3-linked gal-4-SO_4 residue is indicated by the lack of reactivity of the OH^-BH_4 treated λ-carrageenan which contains the 4-linked 3,6 AG but lacks the 3-linked gal-4-SO_4. Kappa and ι-carrageenan differ only in that the latter has a sulphate on C-2 of the 3,6 anhydride. Sulphation of the 4-linked unit (3,6 AG-2-SO_4) reduces the reactivity of the polymer to the anti-κ-carrageenan. Unlike the anti-λ-carrageenan and the anti-κ-carrageenan, the anti-ι-carrageenan preparation does not display specificity to the immunogen used to elicit the antibody (Fig. II,4d), but displays identical specificities to the anti-κ-carrageenan preparations. Two other attempts at immunizing goats to iota-carrageenan failed to elicit specific activity.

CHAPTER III

CHEMISTRY AND IMMUNOCHEMISTRY OF CARRAGEENANS FROM EUCHEUMA AND RELATED ALGAL SPECIES

INTRODUCTION

Unlike certain algal species of the Gigartinaceae algal plants of *Eucheuma* species are known to produce either κ- or ι-carrageenans in both stages (tetrasporic and gametophytic) of their life cycle[26]. Little is known, however, concerning the KCl soluble and insoluble fractions recovered from carrageenan extracts of these species.

We have taken advantage of chemical and immunochemical procedures to elucidate the finer structure of these carrageenans and to obtain further information as to the nature of carrageenan-anti-carrageenan interaction.

RESULTS AND DISCUSSION

Carrageenan Chemistry

Carrageenans of the κ- and ι- types are composed ideally of alternating D-galactose-4-SO_4 and 3,6 anhydro-D-galactose. Iota carrageenan is distinguished from κ-carrageenan by the occurrence of 2-SO_4 on the 3,6 anhydride residue. This pattern may however be interrupted or masked by the occurrence of gal-6-SO_4, gal-2,6-di-SO_4 or even

non-sulphated residues (kinking residues) which causes the molar ratios of gal:3,6AG:SO_4 to deviate from the expected 1:1:1 for κ-carrageenan and 1:1:2 for ι-carrageenan. The KCl insoluble carrageenans contain enough κ- or ι-characteristics (i.e. 3,6 AG or 3,6 AG-2-SO_4) to render them insoluble in KCl. If kinking residues reach a critical proportion relative to the 3,6 anhydride units, the polysaccharides become soluble in KCl[5]. We have fractionated the carrageenans from tetrasporic and gametophytic algal plants of *E. cottonii*, *E. serra*, *E. nudum*, and gametophytic plants of *E. striatum* into KCl soluble and insoluble polysaccharides as described by McCandless *et al.*[28] The proportions of soluble and insoluble carrageenans recovered and their chemical analyses are shown in Table III,1.

Iota-type Carrageenans

Infrared spectroscopy is used regularly to characterize the types of carrageenans obtained from different carrageenophytes. Figures III,1a and 1b shows a portion of the infrared spectra of the various carrageenans used in this study. All the samples exhibit the characteristic ester SO_4 band at 1240-1250 cm^{-1}. The spectra of the KCl soluble and insoluble carrageenans from *E. serra* and *E. nudum* extracted in this laboratory as well

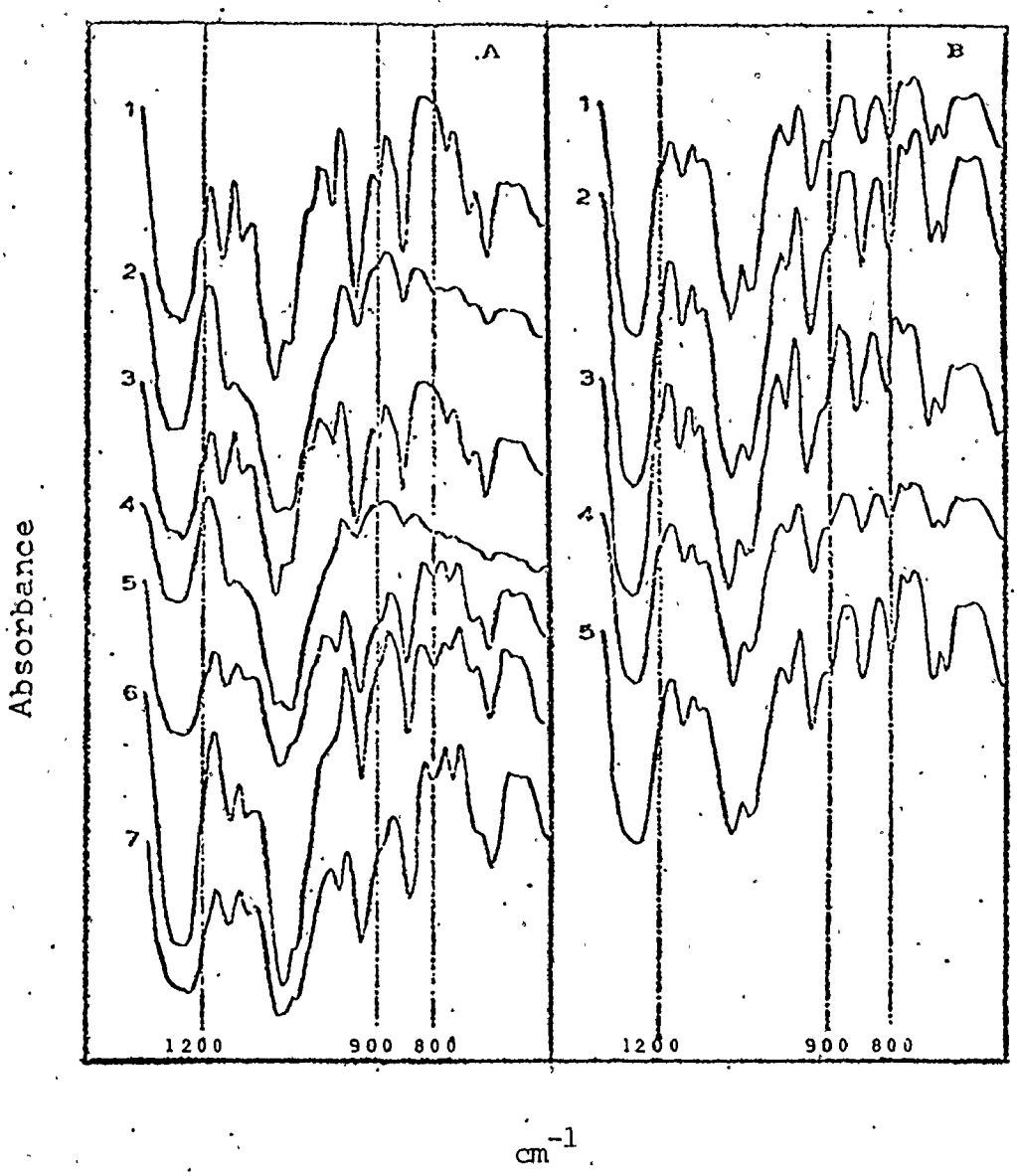

Fig. III,1A: The infrared spectra of κ-carrageenans from 1) *E. cottonii* (female) KCl insoluble; 2) *E. cottonii* (female) KCl soluble; 3) *E. cottonii* (tetrasporic) KCl insoluble; 4) *E. cottonii* (tetrasporic) KCl soluble; 5) *E. striatum* (female) KCl insoluble; 6) *E. striatum* (female) KCl soluble and 7) *C. crispus* (female) KCl insoluble carrageenan.

III,1B: The infrared spectra of ι-type carrageenans from 1) *E. spinosum*; 2) *E. isiforme*; 3) *E. serra*; 4) *E. nudum*; 5) *A. tenera*.

Table III,1: Chemical analysis of *Eucheuma* carrageenans

Sample no.	Source & type of Polysacch.	% Total recovered carrageenan	% SO_4 ± S.E.	%3,6 AG ±S.E.	%3,6 AG increase $\overline{OH}$ BH_4	%3,6 AG increase $IO_4\overline{OH}$ BH_4	Molar ratio gal:3,6AG: SO_4
	E. cottonii ♀						
1	KCl ins.	86.0	20.4 ± 1.5	33.6 ± 1.7	0	0	1:.73:.82
2	KCl sol.	14.0	10.0 ± 0.7	8.2 ± 1.9	4	-	1:.10:.23
	E. cottonii ⊕						
3	KCl ins.	81.4	19.0 ± 1.1	29.5 ± 1.5	0	0	1:.59:.70
4	KCl sol.	18.6	11.5 ± 0.4	6.0 ± 1.4	3	-	1:.07:.27
	E. Striatum ♀						
5	KCl ins.	76.4	20.0 ± 1.6	24.2 ± 1.3	0	0	1:.45:.67
6	KCl sol.	23.6	17.0 ± 0.9	9.2 ± 0.6	-	-	1:.13:.43
	C. crispus ♀						
7	KCl ins.	-	23.1 ± 1.0	28.4 ± 1.6	0	0	1:.59:.89
	E. serra ♀						
8	KCl ins.	62.2	26.5 ± 1.2	17.7 ± 1.0	21	12	1:.32:.89
9	KCl sol.	37.8	26.6 ± 1.5	16.4 ± 1.2	23	26	1:.29:.87
	E. serra ⊕						
10	KCl ins.	66.1	31.0 ± 0.6	18.5 ± 0.9	13	15	1:.38:1.2
11	KCl sol.	33.9	25.0 ± 1.9	15.9 ± 1.5	14	18	1:.27:.79

** Table Continued

Table III,1 (CONTINUED)

Sample no.	Source & type of Polysacch.	% Total recovered carrageenan	% SO_4 ± S.E.	%3,6 AG ± S.E.	%3,6 AG increase $OH^- BH_4$	%3,6 AG increase $IO_4 OH^- BH_4$	Molar ratio gal:3,6AG:SO_4
	E. nudum ♀						
12	KCl ins.	69.0	25.5 ± 1.7	20.9 ± 1.0	7	12	1:.40:.89
13	KCl sol.	31	21.2 ± 2.2	-	-	-	-
	E. nudum ⊕						
14	KCl ins.	67.6	26.7 ± 1.7	20.4 ± 1.4	11	14	1:.39:.95
15	KCl sol.	32.4	23.9 ± 1.3	17.4 ± 0.9	18	19	1:.30:.76
	E. spinosum†						
16	*M.C. Ltd.	-	-	-	18	17	1:.85:1.9
	E. isiforme†						
17	*M.C. Ltd.	-	-	-	26	24	1:.56:1.5
	A. tenera†						
18	*M.C Ltd.	-	-	-	23	18	1:.47:1.4

† As in Lawson *et al.* reference 49

* Sample supplied by Marine Colloids Ltd.

as the carrageenans from *E. spinosum, E. isiforme* and *Agardhiella tenera* all contain the characteristic ι-carrageenan bands (Fig. III,1b). The KCl soluble and insoluble carrageenans from *E. serra* and *E. nudum* (samples 8-15 Table III,1) have similar molar ratios of gal:3,6AG:SO_4. They contain 1.4 to 1.6 SO_4 residues per disaccharide and thus conform closely to ι- type carrageenans. The KCl soluble carrageenans from *E. serra* and *E. nudum* (samples 9,11,13,15 Table III,1) vary slightly from their respective KCl insoluble counterparts (samples 8,10,12,14 Table III,1). The former contain less 3,6 AG and more kinking residues (Table III,2). Whether or not these slight differences can account for the different solubility properties in aqueous KCl is not clear. The majority of the kinking residues of the ι- type carrageenans are of the non-6-sulphated type. Most of the ι-carrageenans (samples 9-15 Table III,2) contain only gal-2,6-di-SO_4 in addition to the non-6-sulphated residues, while samples eight and 16 contain both gal-6-SO_4 and gal-2,6-di-SO_4 kinking residues.

Kappa-type Carrageenans

The infrared spectra of the KCl insoluble carrageenans from tetrasporic and gametophytic plants of *E. cottonii,* and gametophytic plants of *E. striatum* and *C. crispus* show the characteristic κ-type carrageenan bands

Table III,2: Characteristics of 4-linked "kinking" residues

Sample no.	Algal species & nuclear stage	Polysaccharide fraction (solubility in .3 M KCl)	% gal-6-SO_4	% gal-2,6-diSO_4	% non-6-SO_4
1	*E. cottonii* ♀	ins.	0	0	5.4
2	*E. cottonii* ♀	sol.	.4	-	31.0
3	*E. cottonii* ⊕	ins.	0	0	9.6
4	*E. cottonii* ⊕	sol.	.3	-	33.5
5	*E. striatum* ♀	ins.	0	0	14.9
6	*E. striatum* ♀	sol.	0	0	30.2
7	*C. crispus* ♀	ins.	0	0	10.7
8	*E. serra* ♀	ins.	2.5	4.5	10.5
9	*E. serra* ♀	sol.	0	7.9	12.2
10	*E. serra* ⊕	ins.	0	5.0	11.5
11	*E. serra* ⊕	sol.	0	4.8	14.3
12	*E. nudum* ♀	ins.	0	3.0	
13	*E. nudum* ♀	sol.	-	-	-
14	*E. nudum* ⊕	ins.	0	4.5	9.9
15	*E. nudum* ⊕	sol.	0	6.5	11.9
16	*E. spinosum*	Marine Colloids	2.1	4.3	11.9
17	*E. isiforme*	Marine Colloids	0	5.9	11.3
18	*A. tenera*	Marine Colloids	0	3.2	15.1

(Fig. III,1a). These carrageenans contain 0.8 to 0.9 SO_4 residues per disaccharide which conforms to what is expected for κ-type carrageenans. Of these carrageenans, however, only the KCl insoluble carrageenan from gametophytic *E. cottonii* approaches the theoretical molar ratio of 1:1:1 for gal:3,6AG:SO_4. The infrared spectrum of the KCl soluble carrageenan from *E. striatum* is qualitatively similar to the KCl insoluble carrageenan extracted from the same plants. The spectra from the KCl soluble carrageenans from gametophytic and tetrasporic plants of *E. cottonii*, in general, are characteristic of κ-type carrageenans in that they contain 936 cm^{-1} and 840 cm^{-1} bands but differences from either κ- or ι-carrageenan infrared spectra are also evident (Fig. III,1a). The KCl soluble carrageenan from *Eucheuma* species producing κ-type carrageenans differ chemically from their KCl insoluble counterparts. As expected from their solubility properties they contain less 3,6 AG and less SO_4 (Table III,1). They contain a large proportion of kinking residues almost exclusively non-6-sulphated. The KCl insoluble κ-type carrageenans from these species also contain non-6-sulphated type of kinking residues.

Immunochemistry

It was suggested by Hosford and McCandless[21] that

the reactivity of an antiserum to κ-carrageenan was directed to some structural feature associated with 3,6 AG. The antibody preparation from the same antiserum and prepared as described in chapter II demonstrates that the reactivity is in fact not only directed to a structural feature associated with 3,6 AG but that it reacts only when the 3,6 AG is found in association with gal-4-SO_4. If either of these residues is missing in the polymer the antibody will not react[28]. We have also mentioned in chapter II that 2-sulphation of the 3,6 anhydride reduces the reactivity in spite of the presence of gal-4-SO_4. It is possible, therefore to distinguish between κ- and ι-carrageenans and to probe the chemical and structural relations between κ-type carrageenans.

Equivalent amounts of the various carrageenans were reacted with equal amounts of antibody. The interaction of the antigen and antibody was quantified by the agarose medium turbidimetric assay and shown in Fig. III,2. The ability of the antibody preparation to discriminate between κ- and ι-carrageenans is clearly indicated. KCl insoluble carrageenans from tetrasporic and gametophytic plants of *E. cottonii* (Fig. III,2a) and gametophytic plants of *E. striatum* and *C. crispus* (Fig. III,2b) react much better than do the ι-type carrageenans (Fig. III,2b,2c,2d). Table III,3 contains the immunochemical data and the

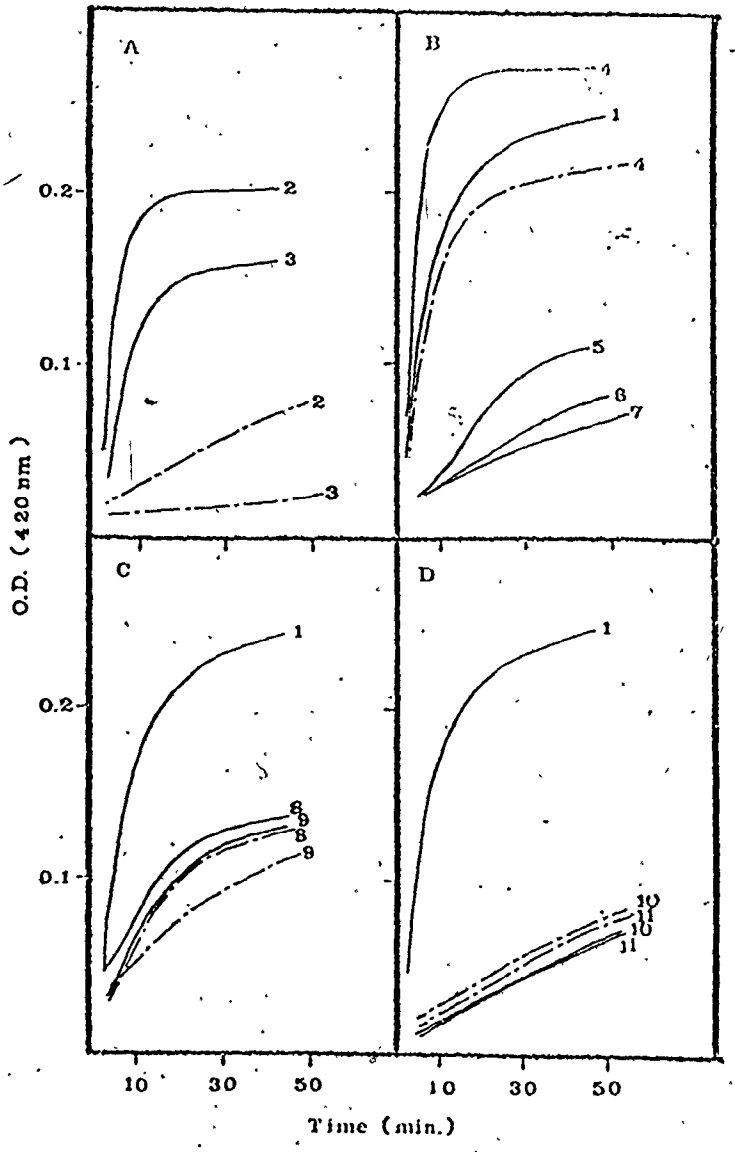

Fig. III,2A-2D: The reactions of KCl soluble (·——·——·) and KCl insoluble (————) carrageenans from 1) *C. crispus* (female); 2) *E. cottonii* (female); 3) *E. cottonii* (tetrasporic); 4) *E. striatum* (female); 5) *E. isiforme*; 6) *E. spinosum*; 7) *A. tenera*; 8) *E. nudum* (female); 9) *E. nudum* (tetrasporic); 10) *E. sera* (female); 11) *E. sera* (tetrasporic), with anti-κ-carrageenan G9-γ-477 as measured by the agarose medium turbidimetric assay.

Table III,3: Immunochemical analysis of carrageenans from *Eucheuma* species.

Sample no	Algal species & nuclear stage	Polysaccharide fraction (solubility in .3 M KCl)	Rate OD/min x 10^3 ± 10%	Total turbidity ± 10%	I.H.
1	*E. cottonii* ♀	ins.	29	0.20	1.3
2	*E. cottonii* ♀	sol.	1.5	0.12	0.04
3	*E. cottonii* ⊕	ins.	15	0.16	0.53
4	*E. cottonii* ⊕	sol.	0	0	0
5	*E. striatum* ♀	ins.	42.5	0.28	2.6
6	*E. striatum* ♀	sol	10.0	0.23	0.9
7	*C. crispus* ♀	ins.	18	0.25	1.0
8	*E. serra* ♀	ins.	1.3	0.10	0.03
9	*E. serra* ♀	sol.	1.3	0.10	0.03
10	*E. serra* ⊕	ins.	1.3	0.10	0.03
11	*E. serra* ⊕	sol.	1.3	0.10	0.03
12	*E. nudum* ♀	ins.	5.5	0.14	0.16
13	*E. nudum* ♀	sol.	2.5	0.13	0.07
14	*E. nudum* ⊕	ins.	4.8	0.13	0.14
15	*E. nudum* ⊕	sol.	5.5	0.13	0.16
16	*E. spinosum*	Marine Colloids	1.6	0.10	0.04
17	*E. isiforme*	Marine Colloids	4.0	0.13	0.10
18	*A. tenera*	Marine Colloids	1.0	0.13	0.03

indices of homology of the various carrageenans. The soluble carrageenans from *E. cottonii* are not only chemically but also immunochemically different from the KCl insoluble carrageenan. The difference between the I.H. of these carrageenans and the homologous antigen cannot be explained by their lower 3,6 AG and SO_4 content only, since the KCl soluble carrageenan from *E. striatum*, although differing chemically from the KCl insoluble carrageenans from *E. striatum* and *C. crispus*, has an I.H. of 0.9 to the homologous antigen. This apparent discrepancy can be easily explained when one considers that only a small proportion of the 3,6 AG residues of the homologous antigen are actually involved in antibody binding[21]. It is suggested that the KCl soluble carrageenan from *E. striatum* differs from the KCl soluble carrageenans from *E. cottonii* in that the 3,6 AG and gal-4-SO_4 residues in the former carrageenan are consecutively arranged in segments sufficiently long to constitute κ-type determinants, whereas in the latter these residues may be dispersed, never occur consecutively, and therefore, the polymers contain few or no κ-type determinants. This is also indicated by the fact that the *E. striatum* carrageenan contains 0.8 SO_4 residues per disaccharide as opposed to 0.42 to 0.50 SO_4 residues per disaccharide in the KCl soluble carrageenans from *E. cottonii*. The KCl soluble carrageenan from

E. striatum is, however, only half as reactive as its KCl insoluble counterpart. The carrageenans from *E. striatum* are, therefore, more efficient at binding antibody than the homologous antigen. This suggests that the κ-type carrageenans from *E. cottonii* and *C. crispus* contain as well, cryptic antibody binding sites (i.e. potential antigenic determinants not available for antibody binding due to the molecular configuration of the particular polymer). In other words, both the content and the availability of antigenic determinants are important in the interaction between antigen and antibody and some differences in the molecular configuration of these carrageenans are suggested.

CONCLUSIONS

The KCl soluble carrageenans from *E. cottonii*, which produces κ-type carrageenans, differs chemically and immunochemically from its KCl insoluble counterpart. The KCl soluble carrageenan from *E. striatum* contains a sufficient proportion of kinking residues to render it soluble in KCl but also contains sufficient κ-type segments to allow significant reactivity with the anti-κ-carrageenan. We postulate that the differences in immunological reactivity between *E. cottonii* and *E. striatum* KCl soluble carrageenans are due to the internal organization of the κ-type

residues.

The ability of some κ-carrageenans to react differently from other κ-type carrageenans suggests the existence of cryptic antibody binding sites in the less reactive carrageenans. Binding sites may be rendered cryptic by the molecular configuration of the particular polysaccharide as determined by the internal organization of kinking residues and κ-type residues. Another possibility is the extent of sulphation of the 3,6 anhydride.

The anti-κ-carrageenan preparation can be used to distinguish κ- from ι-carrageenans. Iota carrageenans show indices of homology of less than 0.2.

CHAPTER IV

IMMUNOCHEMISTRY OF KAPPA-TYPE CARRAGEENANS FROM CERTAIN MARINE RED ALGAE

INTRODUCTION

Carrageenans of the κ-type are composed ideally of alternating D-galactose-4-$SO_4^{\ominus}$ and 3,6 AG units. McCandless *et al.*[27] have demonstrated that κ-carrageenan is restricted to the gametophytic stage of some algal species of the Gigartinaceae. Both KCl soluble and insoluble components of carrageenans extracted from the gametophyte have been shown to exhibit κ-type characteristics[27]. It has been suggested that the KCl soluble component contains the precursor (μ-type structure)to κ-carrageenan[29].

In this chapter we present a study of KCl soluble and insoluble κ-type carrageenans extracted from the gametophytic phase of several species of red algae.

RESULTS

The infrared spectra of the carrageenans used in this study showed marked similarities and Fig. IV,1 shows representative I.R. scans for the carrageenans from *C. crispus*; all contained the general carbohydrate ester SO_4 absorption band at 1240-1250 cm^{-1}. The KCl insoluble carrageenans from

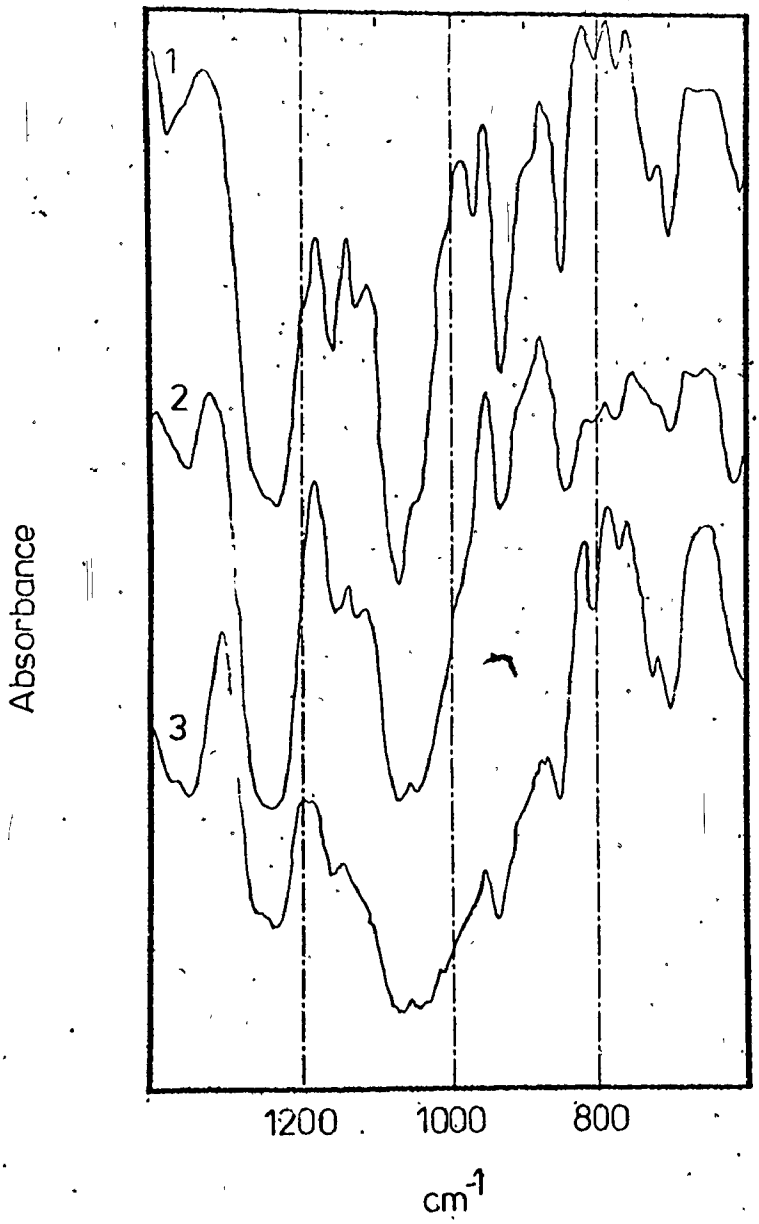

Fig. IV,1: Infrared spectra of 1) KCl insoluble; 2) KCl soluble and 3) alkaline borohydride modified KCl soluble, carrageenans from *C. crispus* (gametophytic).

male and female algal species contained sharp, pronounced absorption bands at 936 and 840 cm^{-1}, indicating the presence of 3,6 AG and gal-4-SO_4 respectively. The KCl soluble carrageenans of gametophytes also showed these κ-type characteristic absorption bands. In general the latter showed less pronounced absorption at 936 cm^{-1} indicating a lower content of 3,6 AG than the KCl insoluble carrageenans, and a broader band with maximum at 840 cm^{-1} attributed to the occurrence of gal-6-SO_4 residues (absorption at 820 cm^{-1}). After OH^-BH_4 treatment the 3,6 AG content of these carrageenans increased (Table IV,1), causing an increase in absorption at 936 cm^{-1} (Fig. IV,1). Due to the elimination of 6-SO_4 in the process of conversion of gal-6-SO_4 to 3,6 AG, a narrowing of the SO_4 absorption band at 840 cm^{-1} was observed (Fig. IV,1). The weak absorption at 805 cm^{-1} (3,6 AG-2-SO_4) observed in these carrageenans also increased in intensity upon OH^-BH_4 treatment (Fig. IV,1).

The SO_4 and 3,6 AG contents of the carrageenans before and after OH^-BH_4 treatment, the latter analysed before and after sodium periodate oxidation, are recorded in Table IV,1.

KCl soluble and insoluble carrageenans from various algae were reacted with an anti-κ-carrageenan the reactivity of which is directed towards 3,6 AG residues associated with gal-4-SO_4[28]. Fig. IV,2 shows the reactivity of the KCl

Table IV,1: Chemical analysis of various carrageenans

Carrageenan source	KCl solubility	% SO_4	% 3,6 AG	µg 3,6 AG/mg carrageenan $OH^- BH_4$ ± S.E.	µg 3,6 AG/mg carrageenan $IO_4^- OH^- BH_4$ ± S.E.	% gal-2,6-diSO_4
C. crispus ♀	ins.	20.0 ± 0.9	21.2 ± 1.9	*	*	-
C. crispus ♀	sol.	23.1 ± 1.0	9.2 ± 1.6	181 ± 2.4	173 ± 5.6	95
I. cordata ♀	ins.	17.1 ± 1.7	17.7 ± 1.8	*	*	-
I. cordata ♀	sol.	26.8 ± 1.9	11.1 ± 1.8	204 ± 6.0	158 ± 9.0	78
R. californicum ♀	ins.	20.2 ± 1.5	20.1 ± 1.8	*	*	-
R. californicum ♀	sol.	20.9 ± 1.9	13.2 ± 1.9	178 ± 1.4	176 ± 6.1	99
R. californicum ♂	ins.	20.1 ± 1.1	19.8 ± 0.3	*	*	-
R. californicum ♂	sol.	21.2 ± 2.9	10.9 ± 2.0	170 ± 3.3	154 ± 8.9	91
G. pistillata ♀	ins.	12.1 ± 1.9	19.2 ± 1.1	*	*	-
G. pistillata ♀	sol.	27.1 ± 1.6	14.5 ± 1.2	238 ± 2.8	227 ± 4.9	95
Gigartina sp. ♂ *(S.F.B.)*†	ins.	18.0 ± 1.6	18.9 ± 1.8	*	*	-
Gigartina sp. ♂ *(S.F.B.)*†	sol.	20.3 ± 1.5	12.8 ± 1.0	224 ± 4.0	195 ± 7.5	88

* No significant change in the level of 3,6 AG after treatment.

† San Francisco Bay

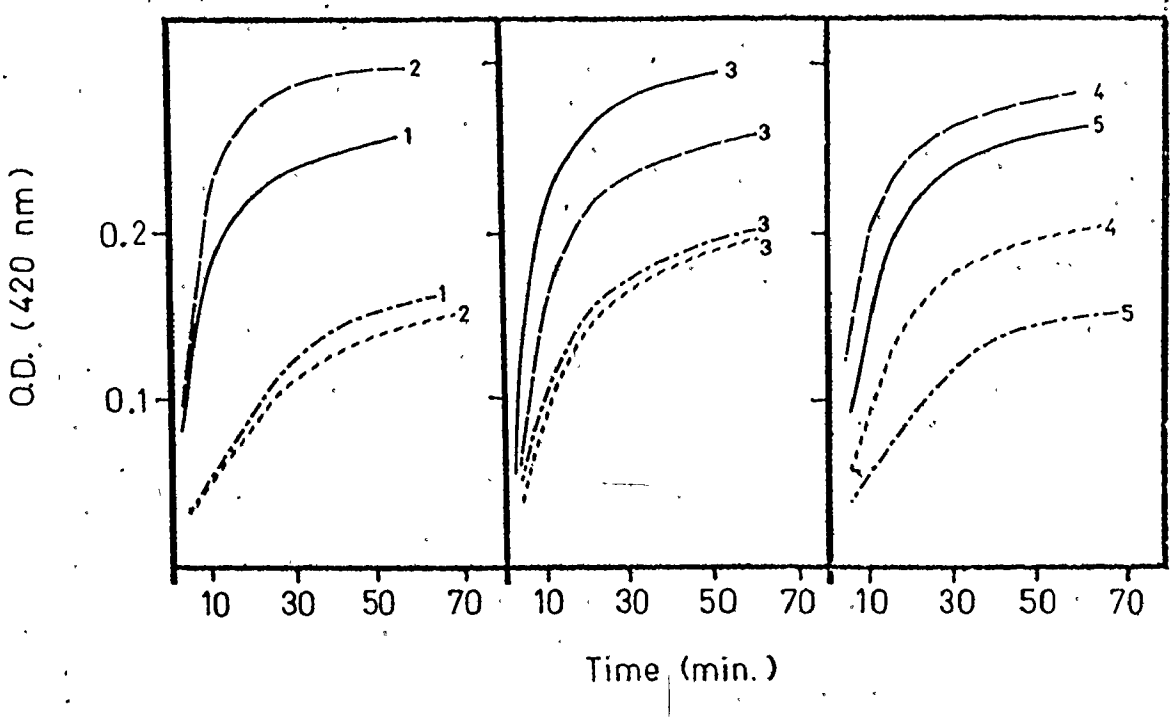

Fig. IV,2: Immunochemical reactivity of KCl insoluble carrageenan from female (————), and male (—·—·—·—) and KCl soluble carrageenan from female (— — — —) and male (- - - - - -) gametophytic algal plants of 1) *C. crispus*, 2) *I. cordata*, 3) *R. californicum*, 4) *Gigartina* species from San Francisco Bay and 5) *G. pistillata*, to anti-κ-carrageenan G9-γ-477 as measured by the agarose medium turbidimetric assay.

soluble and insoluble carrageenans from *Iridaea cordata* (male), *Rhodoglossum californicum* (male and female), a *Gigartina* species from San Francisco Bay (male), *Gigartina pistillata* (female) and *Chondrus crispus* (female). The indices of homology[31] of the various carrageenans to the homologous antigen (KCl insoluble carrageenan from *C. crispus*) are shown in Table IV,2. The reactivity of the KCl soluble carrageenan from *C. crispus*, before and after OH^-BH_4 treatment, to an anti-κ and an anti-λ-carrageenan preparation is shown in Fig. IV,3a and 3b respectively.

DISCUSSION

The infrared spectra of various carrageenans suggests that the KCl soluble carrageenans from the gametophytic algal plants contain 4-linked gal-6-SO_4 residues and less 3,6 AG than the respective KCl insoluble carrageenans. After OH^-BH_4 treatment of the KCl soluble carrageenans, the major contribution to the SO_4 absorption region (800-850 cm^{-1}) is due to gal-4-SO_4 with a maximum at 840 cm^{-1}. The weak absorption at 805 cm^{-1} is enhanced by OH^-BH_4 treatment indicating that a proportion of the increase in the 936 cm^{-1} absorption might be due to the appearance of 3,6 AG-2-SO_4 and not just 3,6 AG. Analysis of 3,6 anhydride content after OH^-BH_4 treatment carried out before and after $NaIO_4$ oxidation (Table IV,1) shows that this is in fact the case;

Table IV,2: Immunochemical analysis of various carrageenans

Carrageenan source	KCl solubility	Rate of precipitation O.D./min x 10^3 ± 10%	Equilibrium O.D. ± 10%	I.H
C. crispus ♀	ins.	11.3	.26	1.0
C. crispus ♀	sol.	4.1	.18	.3
I. cordata ♀	ins.	16.0	.29	1.6
I. cordata ♀	sol.	3.6	.16	.2
R. californicum ♀	ins.	17.9	.31	1.9
R. californicum ♀	sol.	7.3	.20	.5
R. californicum ♂	ins.	12.4	.27	1.1
R. californicum ♂	sol.	7.5	.21	.5
G. pistillata ♀	ins.	10.5	.26	.9
G. pistillata ♀	sol.	3.7	.15	.2
Gigartina sp. ♂ *(S. F.B.)**	ins.	14.4	.28	1.4
Gigartina sp. ♂ *(S. F.B.)**	sol.	6.9	.21	.5

* San Francisco Bay

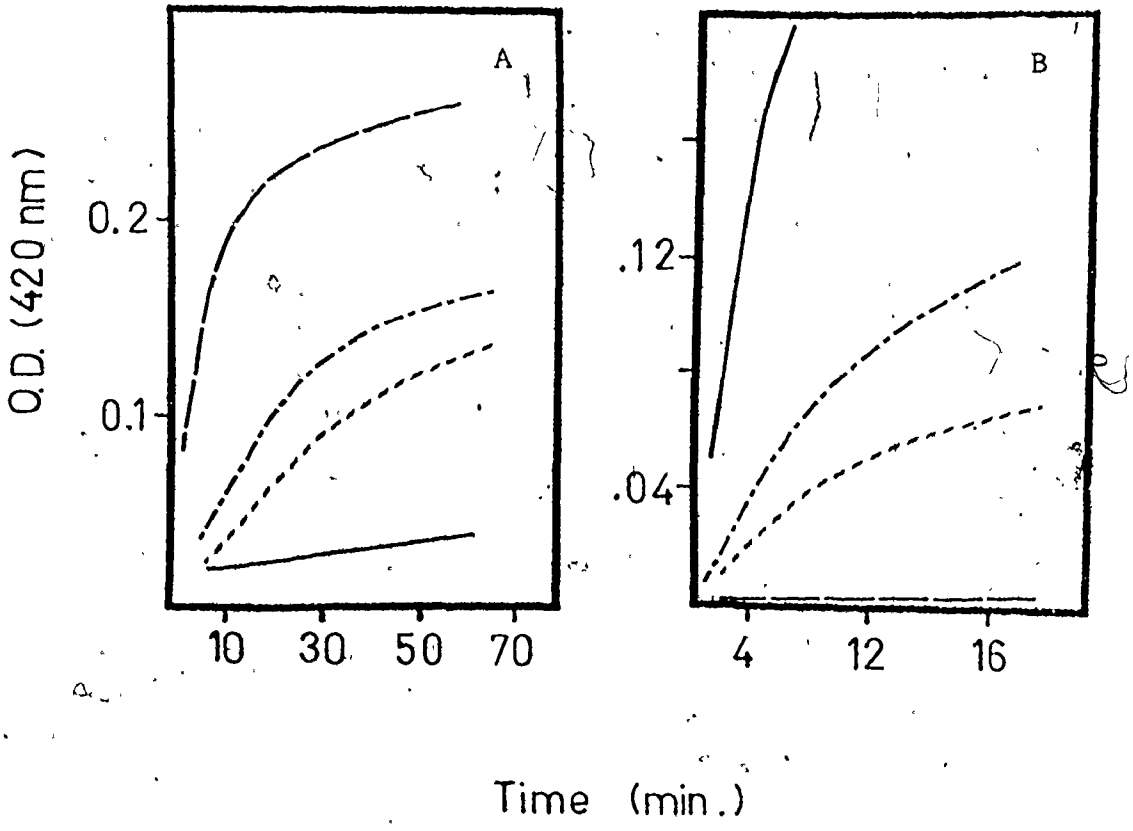

Fig. IV,3: Immunochemical reactivity of KCl insoluble (————), KCl soluble (— · — · —) and alkaline borohydride modified (– – – –) carrageenan from female gametophytic and KCl soluble carrageenan from tetrasporic (————) algal plants of *C. crispus* to A) anti-κ-carrageenan G9-γ-477 and B) anti-λ-carrageenan G7-λ-477, as measured by the agarose medium turbidimetric assay.

78 to 99% of the precursor units to the 3,6 anhydride were resistant to $NaIO_4$ oxidation suggesting that these are gal-2,6-di-SO_4. Alkaline borohydride treatment of KCl insoluble carrageenans caused little change in either the chemical, infrared or immunological data.

Immunochemically the KCl insoluble carrageenans reacted better with an anti-κ-carrageenan than did the KCl soluble carrageenans. The indices of homology of the former (Table IV,2) ranged from 0.9 to 1.9. The KCl soluble carrageenans were less reactive, the I.H. ranging from 0.2 to 0.5. In these carrageenans the lower reactivity may be correlated with higher contents of sulphate (Tables IV,1 and IV,2). The immunochemical variations encountered in the KCl insoluble carrageenans cannot be accounted for by the slight variations in the chemical content of SO_4 or 3,6 AG. The effect of "kinking residues" on secondary structure and the effect of secondary and tertiary structure on the immunochemical reactivity of carrageenans to anti-carrageenans has already been discussed (Chapter III)[10,31,32]. In addition we have observed the occurrence of pyruvated galactose residues in some of the KCl insoluble carrageenans from gametophytic algae (i.e. *G. pistillata*). Although the effect of such residues on carrageenan immunochemistry is unknown they do play an important role in the immunochemical reactivity of bacterial polysaccharides[33].

The KCl soluble carrageenans also show reactivity to an anti-λ-carrageenan (Fig. IV,3b shows the reactivity of carrageenan from *C. crispus*). This indicates the presence of λ-like structures, probably associated with the higher levels of gal-6-SO_4[5,7]. While the chemical data show that after OH^-BH_4 treatment the KCl soluble carrageenans become more similar to κ-type carrageenan (i.e. 3,6 anhydride content increases), immunochemically the reactivity to anti-κ-carrageenan remains low. Their reactivity to the anti-λ-carrageenan decreases as might be predicted by the decrease in 6-SO_4 content[5]. The reactivity of the KCl soluble carrageenans to anti-κ-carrageenan suggests that the preparation contains κ-type molecules which are soluble in KCl and possibly in the process of being synthesized. The chemical data (Table IV,1) show that most of the 3,6 anhydride precursors are gal-2,6-di-SO_4. The alkaline modified KCl soluble polysaccharides, therefore, either represent ι- or alkaline modified λ-carrageenan. Anti-κ-carrageenan reacts poorly with ι- and not at all with alkaline modified λ-carrageenan[28]. McCandless *et al.*[27] showed that OH^-BH_4 treated KCl soluble carrageenan from *C. crispus* could be fractionated into KCl soluble and insoluble fractions. Hosford and McCandless[21] have shown the latter to be reactive with anti-κ-carrageenan serum while the former showed little

reactivity. The anti-κ-carrageenan serum used in that study, however, could not distinguish κ-carrageenan from either ι- or alkaline treated λ-carrageenan. A one step chemical conversion of 6-SO_4 to the 3,6 anhydride is not sufficient to increase the immunochemical reactivity of the KCl soluble carrageenan on alkaline treatment. The theoretical μ as precursor to κ-carrageenan remains elusive and it is suggested by both chemical and immunochemical data that removal of 2-SO_4 on the 4-linked residue may be a necessary step in the synthesis of κ-carrageenan.

CONCLUSIONS

Immunochemically the KCl insoluble carrageenans from the gametophytic algae are highly reactive κ-type carrageenans.

The reactivity of the KCl soluble carrageenans to anti-κ-carrageenan shows the presence of κ-type structures which may represent polymers in the process of conversion to κ-carrageenan. These carrageenans are less reactive to the anti-κ-carrageenan than their respective KCl insoluble carrageenans. The lower reactivity is due to the lower content of κ-type residues (i.e. 3,6 AG), higher levels of 6-SO_4 and the presence of 2-SO_4 on the 4-linked residue.

The reactivity of the KCl soluble carrageenans to

an anti-λ-carrageenan is correlated with the presence of gal-6-SO_4, the unit to which the activity of the anti-λ-carrageenan is directed.

The KCl soluble carrageenans from female plants of the algae *C. crispus*, *R. californicum* and *G. pistillata*, and male plants of *I. cordata*, *R. californicum* and a *Gigartina* species from San Francisco Bay contain less 3,6 AG than the respective KCl insoluble carrageenans. The former also contain high levels of 6-SO_4 and a high proportion of these are found in association with 2-SO_4 (4-linked gal-2,6-di-SO_4).

Both the chemical and the immunochemical data suggest that the increase in 3,6 anhydride on alkali treatment is not translated to an increase in κ-type molecules and that synthesis of κ-carrageenan in the *Gigartinaceae* may not be mediated, simply, through the enzymatic removal of 6-SO_4 from μ-carrageenan which to date remains an elusive polymer.

CHAPTER V

IMMUNOCHEMISTRY OF LAMBDA TYPE CARRAGEENANS FROM CERTAIN MARINE RED ALGAE

INTRODUCTION

The demonstration that in some members of the Gigartinales, κ-carrageenan is restricted to the gametophyte and λ-carrageenan to the sporophyte[27] has led to the preparation of purer κ- and λ-carrageenans.

In this chapter we present a quantitative immunochemical study of carrageenans extracted from tetrasporic stages of several species of algae.

RESULTS

Characteristics of the anti-λ-carrageenan

A commercial preparation of λ-carrageenan supplied by Marine Colloids Inc. was subjected to controlled alkaline borohydride modification in order to obtain samples with varying amounts of galactose-6-SO_4. Table V,1 indicates the sample notations and the 3,6 AG content of the samples. An increased content of 3,6 AG is concomitant with a decreased content of gal-6-SO_4 since 3,6 AG is formed by S_N2 elimination of 6-SO_4. The ability of each sample to precipitate anti-λ-carrageenan is shown in Fig. V,1. A

marked decrease in reactivity is noted with increased modification.

Table V,1. Controlled alkaline borohydride modification of λ-carrageenan

Carrageenan sample	Duration of OH^-BH_4 treatment	% 3,6 AG
1	0	0.35
2	1/2 h	6.4
3	1 h	10.3
4	1 1/2 h	13.1
5	4 h	16.9
6	7 h	19.1

Immunochemical Analysis of Carrageenans

Equivalent amounts (25 µg) of various KCl soluble carrageenans were reacted with anti-λ-carrageenan. Fig. V,2 shows the reactivity of KCl soluble carrageenans from *C. crispus, R. californicum, I. cordata, G. corymbifera, Gigartina* sp. from San Francisco Bay, and *P. middendorfii (franciscana)* tetrasporophytes before and after OH^-BH_4 modification. The carrageenans from *I. cordata* and *R. californicum* show lower reactivity to the anti-λ-carrageenan than the homologous antigen (i.e. *C. crispus* carrageenan),

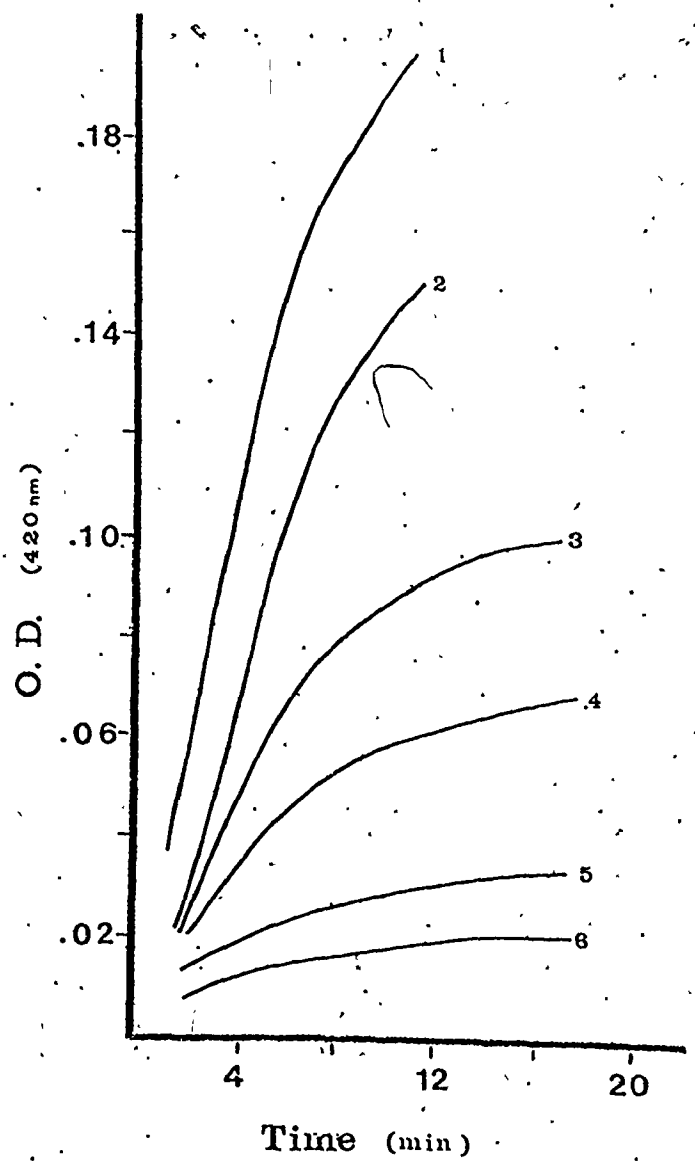

Fig. V,1: Agarose medium turbidimetric assay of the reactivity of λ-carrageenans with varying degree of alkaline borohydride modification (see Table V,1) to an anti-λ-carrageenan G7-γ-477.

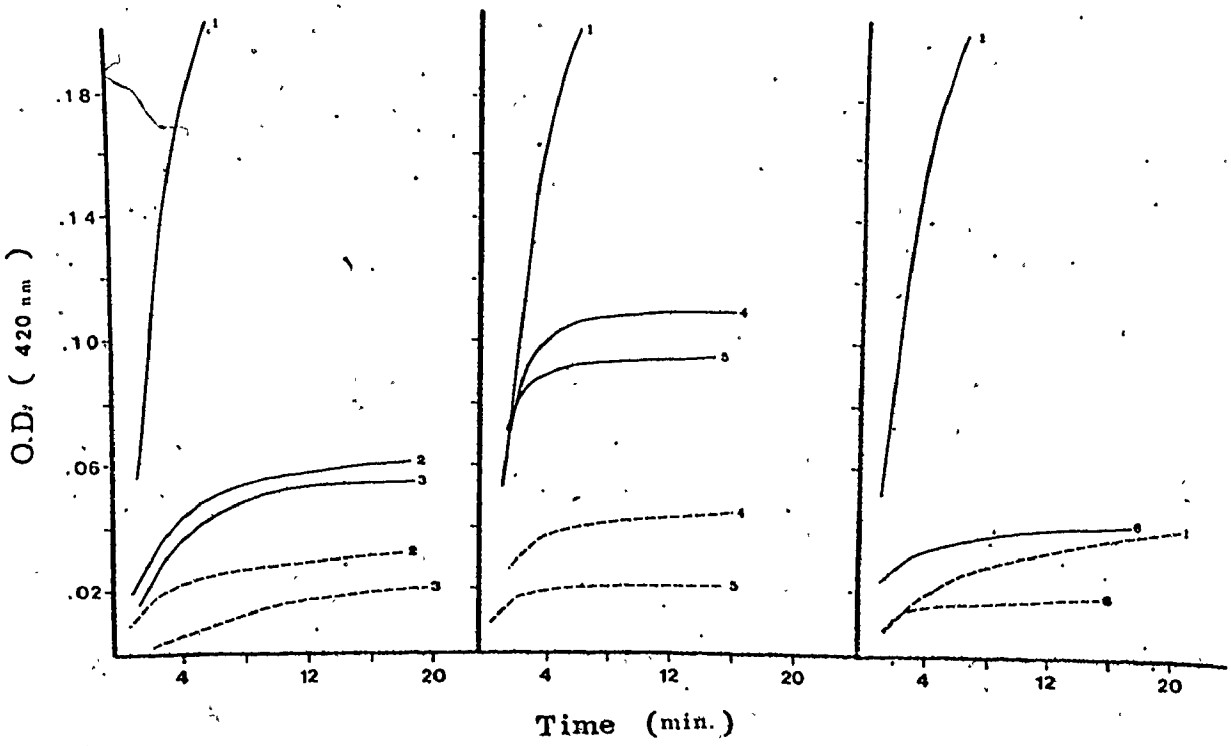

Fig. V,2: Immunochemical reactivity of KCl soluble carrageenan from 1) *C. crispus*, 2) *Gigartina* species from San Francisco Bay, 3) *G. corymbifera*, 4) *R. californicum*, 5) *I. cordata* and 6) *P. middendorfii* (*franciscana*) before (————) and after (– – – – – –) alkaline borohydride treatment, to an anti-λ-carrageenan G7-γ-477 as measured by the agarose medium turbidimetric assay.

while the *Gigartina* and *Petrocelis* show very little reactivity. In all cases a further decrease in Ag-Ab reactivity is noted on alkaline borohydride modification. Table V,2 shows the indices of homology of the various carrageenans.

Chemical Analysis of Carrageenans

The infrared spectra of the carrageenans used in this study are presented in Fig. V3. These infrared spectra show marked similarities in that all contain the general carbohydrate ester-SO_4 band at 1240-1250 cm^{-1}. The infrared spectra of *C. crispus, R. californicum,* and *I. cordata* carrageenans show a broad band between 800 and 850 cm^{-1} with a peak at 827 cm^{-1}. After OH^-BH_4 treatment the broad peak at 827 cm^{-1} is resolved into an 805 cm^{-1} peak representing 3,6 AG-2-SO_4 and an 830 cm^{-1} peak for the *C. crispus* KCl soluble carrageenan with a concomitant appearance of the 936 cm^{-1} peak due to the 3,6 anhydride. For *I. cordata* and *R. californicum* the band remains broad between 800 and 960 cm^{-1} but the peak absorption is shifted to 835 cm^{-1} indicating a change in the proportions of primary, secondary axial and secondary equatorial ester sulphate groups; a peak at 936 cm^{-1} also appears after OH^-BH_4 treatment. The spectra of the two *Gigartina* species show a narrow band peaking at 830 cm^{-1} which remains

Table V,2: Immunochemical analysis of λ-type carrageenans

Source of carrageenan†	Carrageenan type	Equilibrium turbidity ± 10%	Rate of precipitation O.D./min x 10^3 ± 10%	I.H.
C. crispus	λ	.24	46	1.0
C. crispus	λ*	.05	4.6	.02
R. californicum	λ	.11	40	.40
R. californicum	λ*	.04	12	.05
I. cordata	λ	.10	45	.40
I. cordata	λ*	.02	12	.03
G. corymbifera	λ	.06	9.8	.05
G. corymbifera	λ*	.02	2.0	.01
Gigartina sp. (S.F.B.)§	λ	.06	8.0	.05
Gigartina sp. (S.F.B.)§	λ*	.03	3.4	.01
P. middendorfii	λ	.04	12	.05
P. middendorfii	λ*	.02	5.6	.01

* alkaline borohydride treated sample
† tetrasporic algal plants
§ San Francisco Bay

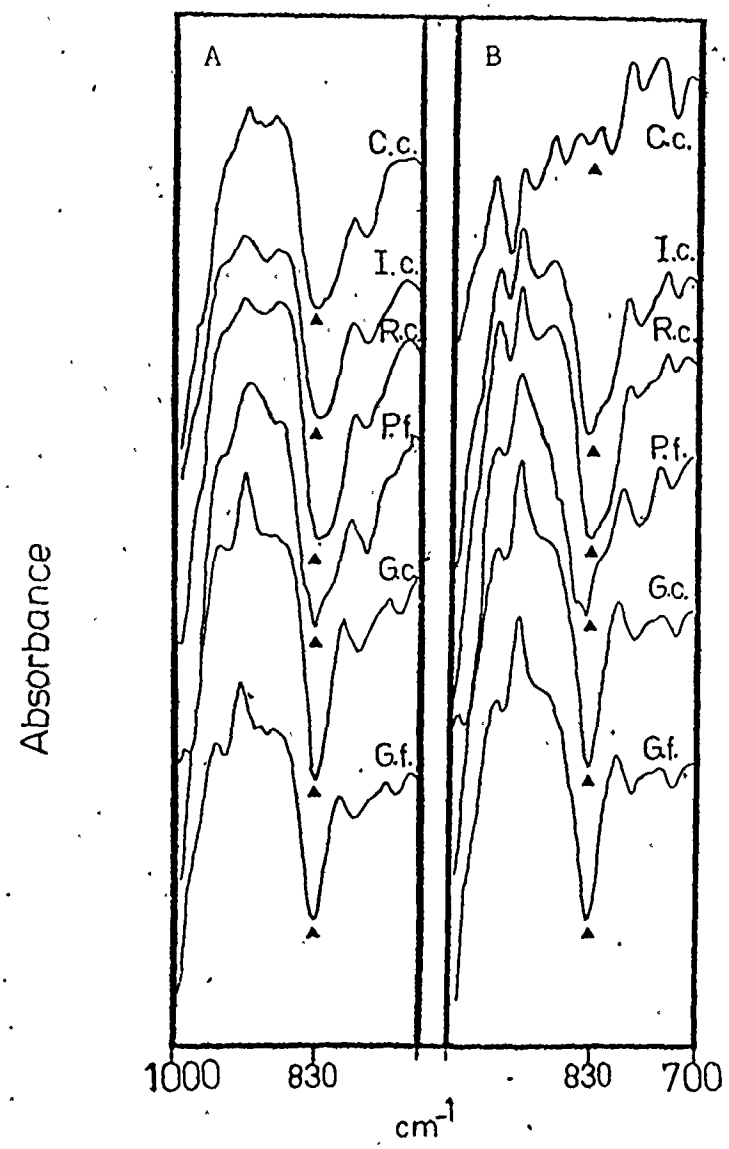

Fig. V,3: The infrared spectra of "λ" type carrageenan from various algal species before (A) and after (B) alkaline borohydride treatment.

relatively unchanged after alkaline borohydride treatment. The *P. middendorfii* carrageenan shows a broad peak between 800 and 850 cm^{-1} with a sharp peak at 830 cm^{-1}. After alkaline borohydride treatment the band remains broad with peaks at 835 and 845 cm^{-1}.

The analysis of 3,6 AG content before and after OH^-BH_4 treatment, the latter analyzed before and after $NaIO_4$ oxidation, is recorded in Table V,3. The resistance of all these polymers to $NaIO_4$ demonstrates that all the precursors to the 3,6 anhydride ar gal-2,6-di-SO_4 (Table V,3). The *Gigartina* and *Petrocelis* carrageenans showed lower 3,6 anhydride content after OH^-BH_4 treatment than the *C. crispus, R. californicum* and *I. cordata* carrageenans, indicating a lower content of 6-SO_4 in the former carrageenans. The *I. cordata* and *R. californicum* carrageenans have similar levels of 6-SO_4 than the *C. crispus* carrageenan.

Gas-liquid Chromatography

The monosaccharide content of *G. corymbifera, Gigartina* sp. from San Francisco Bay, *P. middendorfii* and *C. crispus* were analysed by glc of the trifluoroacetate derivatives of the O-methyl-glycosides obtained by methanolysis. The results are reported in Fig. V,4. Variations in the monosaccharide content and therefore, differences in primary structure

Table V,3: Chemical analysis of λ-type carrageenans

Source of λ-type carrageenan	µg 3,6 AG/mg carrageenan ± S.E.	µg 3,6 AG/mg carrageenan OH^-BH_4 ± S.E.	µg 3,6 AG/mg carrageenan $IO_4^-OH^-BH_4$ ± S.E.	% gal-2,6-diSO_4
C. crispus	20 ± 1.4	170 ± 2.5	164 ± 2.0	96
R. californicum	13 ± 2.4	164 ± 1.2	162 ± 1.1	100
I. cordata	6 ± 0.9	191 ± 4.0	209 ± 3.6	100
G. corymbifera	40 ± 1.4	96 ± 2.9	91 ± 1.7	94
*Gigartina sp. (S.F.B.)**	55 ± 4.2	108 ± 1.8	102 ± 2.0	94
P. middendorfii	30 ± 1.7	85 ± 1.5	91 ± 2.0	100

* San Francisco Bay

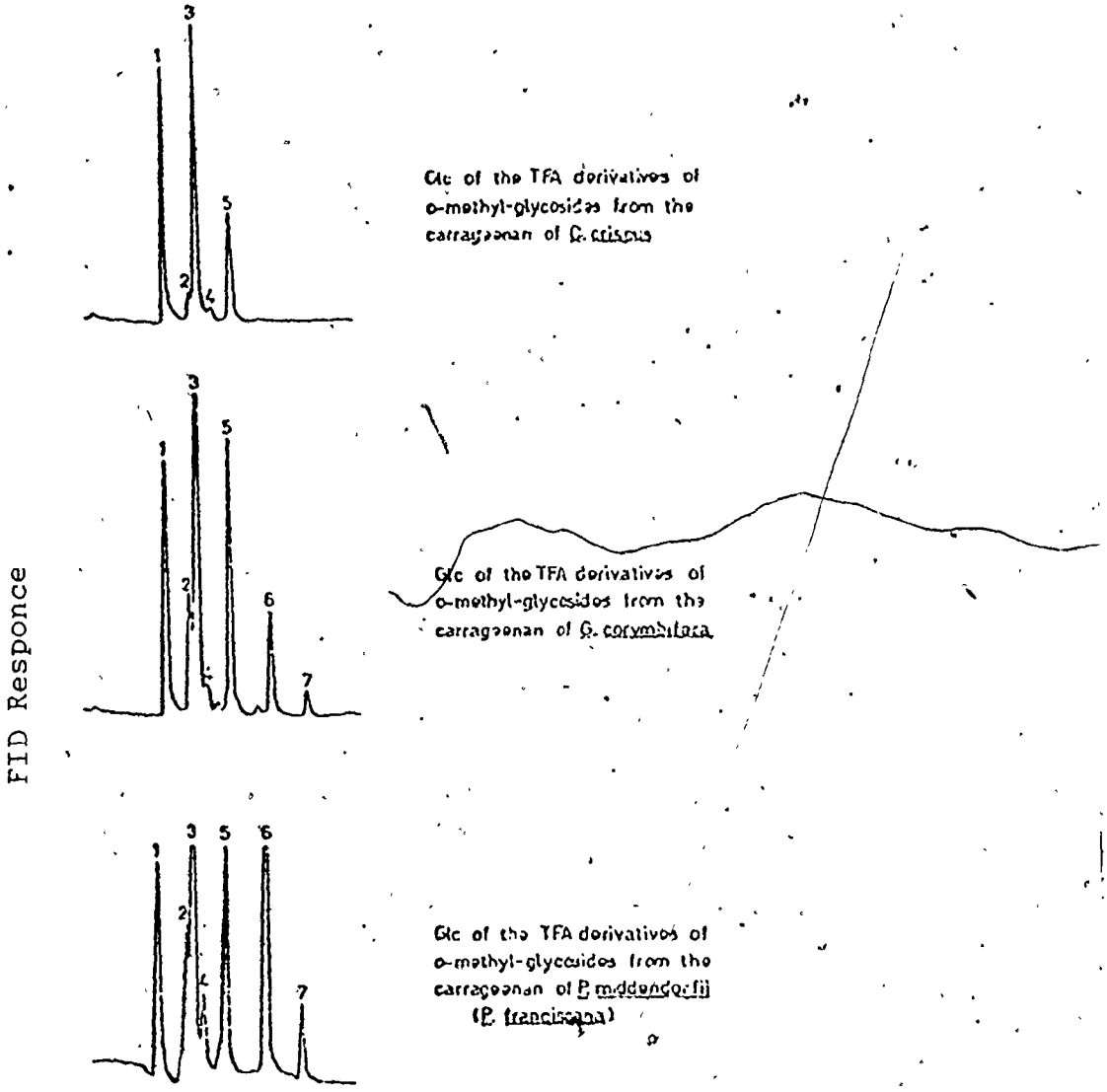

Fig. V,4: Gas-liquid chromatography of the O-methyl-glycosides (TFA derivatives) from the λ-type carrageenans from *C. crispus*, *G. corymbifera* and *P. middendorfii*.

* glc of *Gigartina* sp. (S.F.B.) is identical to that of *G. corymbifera*.

are detected. Of particular interest is the detection of a compound (Fig. V,3, peaks number 6 and 7) whose retention characteristics correspond to the trimethylsilylated (TMS) derivative of 4,6-O-(1-carboxyethylidene)-D-gal [4,6-O-(1-CE)-D-gal] as described by Hirase and Watanabe[38] for a polysaccharide from *G. tenella*, in the carrageenans from *G. corymbifera* and a *Gigartina* species from San Francisco Bay. High levels of this compound were detected in the *P. middendorfii* carrageenan.

DISCUSSION

While the immunochemical method may be used to detect quantitative differences in certain specific chemical features of antigens[33] the actual antigen-antibody reaction is not determined solely by primary structure but represents a complex stereochemical interaction in which slight modification or variations in primary, secondary and tertiary structures may have pronounced effects on the interaction between antigen and antibody. The reactivity of λ-carrageenans to anti-λ-carrageenan is greatly influenced by OH^-BH_4 modification of the polymer (fig. V,1). Whether the decreased reactivity to the antibody is due to the loss of 6-SO_4 or to the secondary and tertiary conformational changes produced by the introduction of the 3,6 anhydride cannot be determined precisely. It is clear, however, that some structural feature associated with

gal-6-SO_4 or possibly gal-2,6-di-SO_4 is involved in antigen-antibody interaction. The 6-SO_4 structural feature represents the major but not the sole determining factor since even after exhaustive OH^-BH_4 modification the resulting polymer still shows some reactivity to the anti-λ-carrageenan.

Immunochemically the KCl soluble carrageenans from *R. californicum* and *I. cordata* have closer homology to *C. crispus* than the carrageenans from either of the *Gigartina* species and *P. middendorfii (franciscana)*. The extremely low indices of homology of the *Gigartina* and *Petrocelis* carrageenans may be due to the lower content of gal-2,6-di-SO_4 and the ensuing conformational alteration. These carrageenans and in particular the *Petrocelis* carrageenan contain a monosaccharide component not detected in the carrageenans from *C. crispus, I. cordata* and *R. californicum*. This component is thought to be 4,6-O-(1-CE)-D-gal. The identification of this compound will be discussed in the subsequent chapter as glc retention times are not definitive proof for the occurrence of a particular compound.

The carrageenans from *I. cordata* and *R. californicum* show an index of homology of 0.4. While the content of 6-SO_4 in these polysaccharides is as high as that of the *C. crispus* carrageenan, and while they show a higher I.H. than the *Gigartina* and *Petrocelis* species carrageenans, some other structural difference must be responsible for the fact that the I.H. is less than 1. This structural feature need not

be associated with 6-SO_4

Using immunodiffusion with an absorbed anti-λ-antisera and infrared spectroscopy, Evelegh *et al.*[34] and McCandless *et al.*[35] have described qualitative differences in the same carrageenans. These were interpreted as being due to the lack of 6-SO_4 in the *Gigartina* carrageenans. In the present study the author has further confirmed that the anti-λ-carrageenan reactivity (chapter II) is directed against some structural feature associated with 6-SO_4. The close similarity of the carrageenans from the *Gigartina* species discussed here to ξ-carrageenan from *G. chamissoi*, *G. canaliculata* and *G. atropurpurea* described by Penman and Rees[5] is indicated by the low I.H. relative to *C. crispus* carrageenan, by the lower content of 6-SO_4 and by the differences in the infrared spectra. Since these carrageenans do contain enough 6-SO_4 to produce 5% 3,6 anhydride on OH^-BH_4 treatment which is accompanied by a decrease in immunological reactivity to the anti-λ-carrageenan, these may in fact represent hybrid ξ-λ-carrageenan molecules, although the possiblity that λ-carrageenan might occur as a separate component cannot be ruled out. The infrared spectrum of the *Petrocelis* carrageenan after alkaline borohydride modification is unusual in that it contains an absorption band at 845 cm^{-1}. The presence of high levels of 4,6-O-(1-CE)-D-gal in the latter carrageenan may contribute to the low immunochemical reactivity.

The significance of this compound to carrageenan structure and algal taxonomy will be discussed in the subsequent chapter.

We conclude that immunochemistry used in conjunction with infrared spectroscopy and simple chemical analysis is useful in detecting and elucidating structural variations which occur in certain related classes of carrageenans.

CHAPTER VI

PYRUVATED CARRAGEENAN FROM *P. MIDDENDORFII*

"π-CARRAGEENAN"

INTRODUCTION

The pyruvated acid ketal 4,6-O-(1-CE)-D-gal is a widespread component of bacterial[36] and algal[37,38] polysaccharides. This structural component has been reported to play an important role in the antigenic makeup of bacterial polysaccharides[36].

This residue is normally identified by hydrolytic removal of the pyruvic acid from the polysaccharide and its detection as the crystalline 2,4-dinitrophenylhydrazone[39]. The linkage of the pyruvic acid to the carbohydrate is then determined by complex and tedious chemical manipulations.

We report, in this chapter, a high content of pyruvic acid in the carrageenan from *P. middendorfii* (*franciscana*). Gas-liquid chromatography of the trifluoroacetic acid (TFA) derivatives of the O-methyl-glycosides obtained by methanolysis of the carrageenan suggested that the pyruvic acid occurred as the pyruvic acid ketal of the galactose in the polymer[38,7]. The retention time, however, is not sufficient for definitive identification of this compound since several compounds may have identical retention times. Since important structural

features of monosaccharides can be determined through their mass-spectrum, the use of combined glc/ms should prove a relatively simple approach to the detection and identification of 4,6-O-(1-CE)-D-gal in biopolymers.

RESULTS AND DISCUSSION

The carrageenan from *P. middendorfii (franciscana)* collected at Mission Pt., Monterey Co., Calif., was extracted as described by McCandless *et al.*[27]. This carrageenan contains 32% SO_4 and essentially no 3,6 AG and upon treatment with $OH^- BH_4$ the 3,6 AG content increased to only 5% (Table V,3). The significance of this to the sulphation pattern and immunochemical reactivity to an anti-λ-preparation has already been discussed (chapter V)[32].

Pyruvic acid was detected in these carrageenans by the enzymatic procedure of Duckworth and Yaphe[40] and supported by proton magnetic resonance (PMR) spectroscopy; the PMR spectrum of the methanolysate in DMSO, showed the presence of a single methyl peak at δ1.44 ppm in agreement with the assignment given by Morris *et al.*[41] for pyruvate in a xanthan. The pyruvate content was estimated to be 6.5% by PMR of the native polysaccharide. The pyruvated acid ketal 4,6-O-(1-CE)-D-gal was first reported in agar by Hirase[42] and was subsequently reported to occur in low concentration in agars prepared from a number of agarophytes[37], and in microbial polymers[36]. The

same acetal has also been demonstrated in a carrageenan extracted from the marine red alga *G. tenella*[38]. The carrageenan contained 1.5% pyruvic acid, an amount equal to one pyruvate residue in twenty sugar residues. To date this has remained an isolated report.

To determine whether the pyruvate in the *Petrocelis* carrageenan occurred as the acid ketal 4,6-O-(1-CE)-D-gal, the carrageenan was submitted to methanolysis and TFA derivatives of the O-methyl-glycosides were prepared. The TFA derivatives were analysed by glc. The results of the glc are shown in fig. VI,1 and indicate the presence of TFA-methyl-galactosides (retention times 3.94, 4.42, and 6.05; $R_{myoinositol}$ 1.39, 1.56 and 2.14 respectively) and substituted TFA-methyl-galactosides (retention times 9.51 and 13.53; $R_{myoinositol}$ 3.36 and 4.78 respectively), the retention characteristics of which correlate closely with those described by Hirase and Watanabe[38] for an authentic sample of the TMS derivatives of 4,6-O-(1-CE)-D-gal. In this sample, however, the TFA-methyl-galactosides and TFA-methyl-4,6-O-(1-CE)-D-galactosides occurred in a molar ratio of 2:1 which represents one pyruvated galactose /1.5 disaccharide units.

To obtain definitive proof of the presence of the acid ketal 4,6-O-(1-CE)-D-gal the TFA derivatives of the methanolysates were separated by glc on an OV-17 Chromosorb W column and fed directly into a mass spectrograph. The resulting

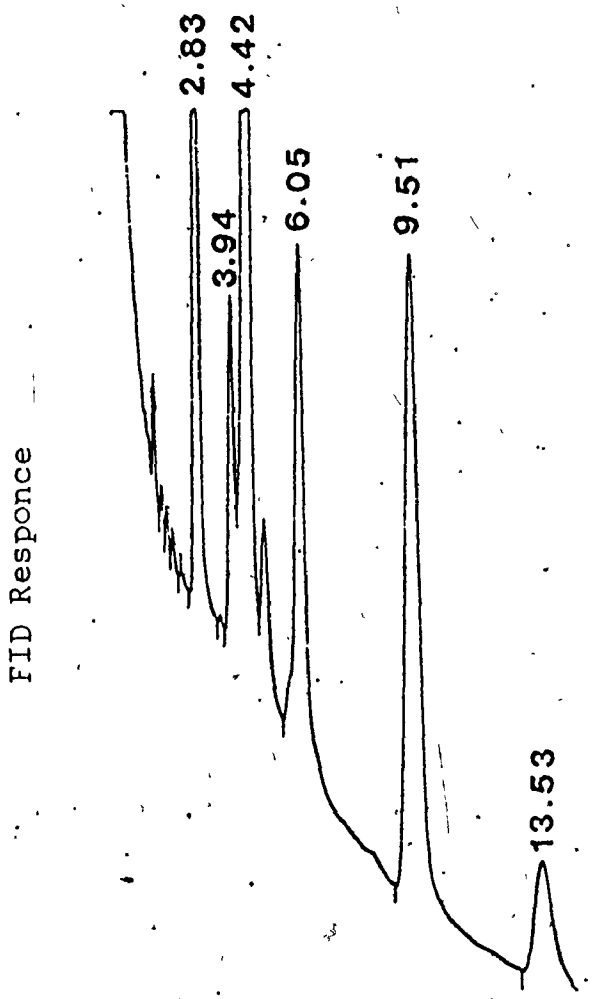

Fig. VI,1: Gas-liquid chromatography of the TFA derivatives of the O-methyl-glycosides from *Petrocelis* carrageenan. Retention time of 2.83 min is that of the internal standard myoinositol. Retention times of 3.94, 4.42 and 6.05 min correspond to TFA-methyl galactosides and retention times of 9.51 and 13.53 min correspond to the TFA-methyl-4,6-O-(1-CE)-D-galactosides.

fragmentation pattern of the compound with $R_{myoinositol}$ 3.36 (fig. VI,1) was again consistent with that expected for 4,6-O-(1-CE)-gal (TFA-methyl derivative) [43]. The mass spectral fragmentation pattern of the compound with retention time similar to 4,6-O-(1-CE)-D-gal is presented in figs. VI,2,3,4. Only those fragments of high mass ($\geq$ 180) are considered since the larger fragments are characteristic and therefore diagnostic of the parent species. It is clearly indicated in fig. VI,2 that the pyruvic acid ketal serves as an effective electron source and directs the major fragmentation of the molecule in such a manner as to leave its position between oxygens 4 and 6 mandatory. The parent ionic species (m/e 470) was indistinguishable from background presumably because loss of the carbomethoxy group ($\cdot COOCH_3$, mass 59) leads to the highly stabilized ketal cation (m/e 411, I/B 100%). The subsequent fragmentation of this ion leads readily to a majority of other high mass fragments (m/e = 351,319,237 and 195) and the sequence shown in fig. VI,2 clearly shows the directing effect of the ketal cation in the progressive disruption of the molecule.

The more normal fragmentation pathways initiated by electron loss from the O ring atom were also found and the ions m/e 337, 223 and 109 are accounted for in fig. VI,3 and ion m/e 183 is rationalized in fig. VI,4.

Other ions in the mass spectrum can be similarly

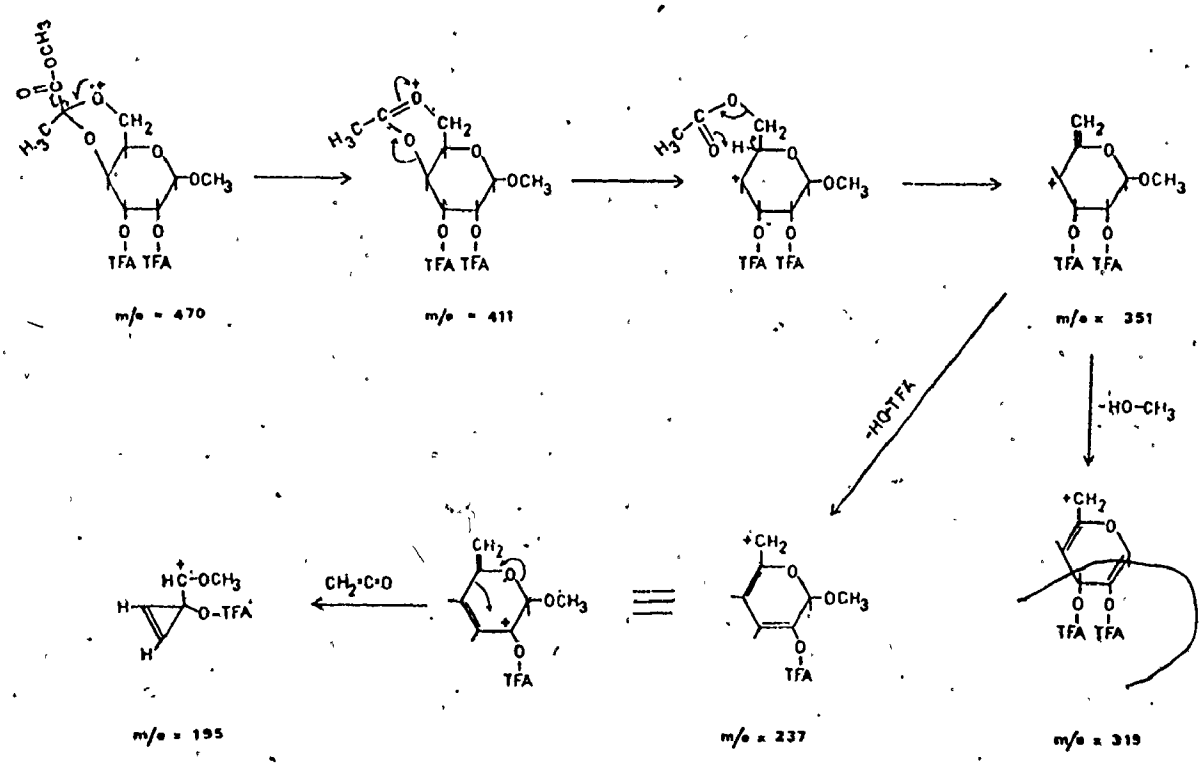

Fig. VI,2: Fragmentation scheme rationalizing the m/e = 411 (I/B 100%); m/e = 351 (I/B 11.9%); m/e = 319 (I/B 16.9%); m/e = 237 (I/B 32.7%); and m/e = 195 (I/B 40.4%) ionic species.

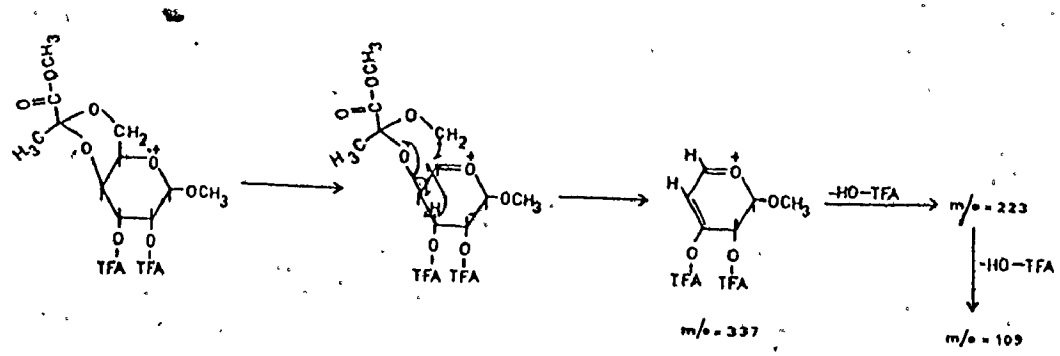

Fig. VI,3: Rationalization of m/e = 337 (I/B 4%); m/e = 223 (I/B 11.4 %) and m/e = 109 (I/B 20.5%) ionic species.

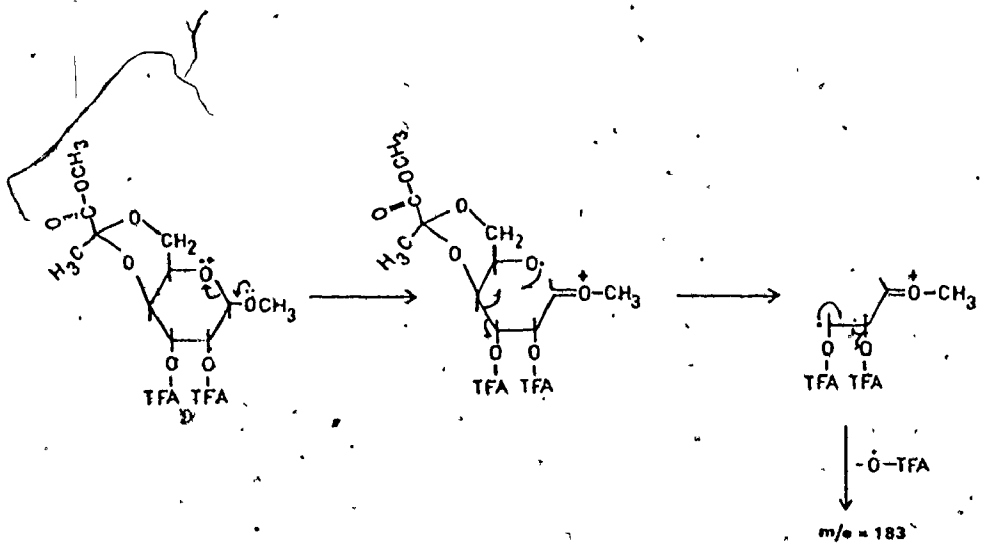

Fig. VI,4: Rationalization of the m/e = 183 (I/B 14.4%) ionic species.

rationalized following normal rules; those presented are more than adequate to characterize 4,6-O-(1-CE)-D-gal and they define precisely the position of the acid ketal unit. Combined glc/ms, therefore, offers a simple rapid method for definitive identification and location of this important carbohydrate substituent.

We have also found the same unit, in lower concentrations, only in carrageenans from several other *Petrocelis* species and related *Gigartina* species. Recent evidence suggests that *P. middendorfii (franciscana)* represents the sporophyte generation of *G. papillata*[42]. Thus, the occurrence of pyruvate in these carrageenans may have taxonomic significance.

Unlike agar, in which the pyruvate has been reported to occur in a fraction with little SO_4 or remote from the sulphated regions of the molecules[45] the polysaccharide from *P. middendorfii* is composed entirely of sulphated residues and the pyruvate must occur in association with these sulphated sugar residues. Since the backbone of carrageenan molecules consists of 1,3 β and 1,4 α-D-galactosides (i.e α-1,3 and β-1,4 linked) we propose that the carrageenan from *P. middendorfii* contains a masked repeating structure consisting primarily of alternating residues of 1,3-linked 4,6-O-(1-CE)-β-D-gal-2-SO_4 and 1,4 linked α-D-gal-2-SO_4. The major component thus represents a new carrageenan species

which we propose to call π-carrageenan (fig. VI,5.). A small proportion of 3-linked β-D-gal-2-SO_4 and 4-linked α-D-gal-2,6-di-SO_4 units are present. It is impossible to state whether these are dispersed among the π-carrageenan units or whether they represent a contaminating λ-carrageenan.

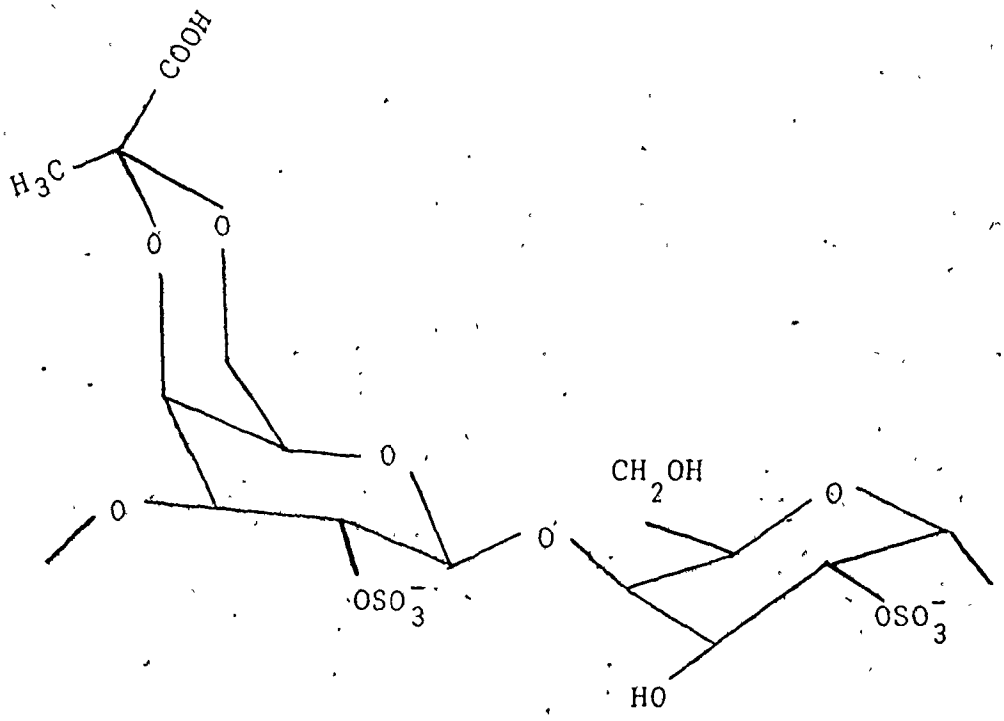

Fig. VI, 5: The major repeating unit of π-carrageenan.

SUMMARY

1.) Appropriate concentrations of carrageenan and anti-carrageenan antibody were mixed in a medium consisting of 0.1% agarose in 0.01 M phosphate buffered saline pH 7.4 at 37°. The kinetics of the precipitation reactions were followed by turbididmetric analysis using a double beam Unicam spectrophotometer at 420 nm. The addition of agarose enhanced the reaction between antigen and antibody as judged by turbidity and served as a stabilizing medium permitting extended periods of continuous monitoring. The initial rate of precipitation was found to vary linearly with the concentration of antigen in the antibody excess zone. Both the initial rate of precipitation and the final optical density were characteristic of the specific antigen-antibody reaction. These two parameters are used to quantitate the extent of cross-reaction and to calculate an index of homology for the cross-reacting antigen to the homologous antigen.

2.) The component(s) responsible for carrageenan interaction with normal serum are distinct from the γG-globulin fraction induced by immunization. The component(s) is not C-reactive protein as determined by the lack of reactivity of both human and mouse C-reactive protein in

Ouchterlony immunodiffusion plates. The pre-immune reactivity is identified with the water insoluble euglobulin fraction of serum.

3.) The activity of the anti-λ-carrageenan (G7-γ-477) is specific for λ-carrageenan. It is suggested that the reactivity of the anti-λ-carrageenan is directed towards a structural feature associated with the 6-SO_4 of the 4-linked residue.

The anti-κ-carrageenan preparations (G9-γ-477) and (G15-γ-777) are specific for κ-carrageenan with a small amount of cross-reactivity with ι-carrageenan. It is suggested that the activity of the antibody is directed to some structural feature assosiated with 4-linked 3,6 AG residues. It reacts only with those polymers which contain this residue in association with 3-linked gal-4-SO_4. If either of these components is missing the polymer will not react. It is also indicated that sulphation at C-2 of the 3,6 anhydride significantly reduces antigen-antibody reactivity in spite of the presence of the 3-linked gal-4-SO_4.

We have been unable to produce specific antibody to ι-carrageenan. The anti-ι-carrageenan preparation described in chapter II displays identical specificities to the anti-κ-carrageenan preparations.

4.) Carrageenans from several species of *Eucheuma* were fractionated into KCl soluble and insoluble fractions and were analysed by the usual chemical procedures. An anti-κ-carrageenan (G9-γ-477) was used to analyse these carrageenans immunochemically. The antibody preparation shows only a small amount of cross-reactivity with the ι-type carrageenans and thus could be used to distinguish κ and ι-type carrageenans, the latter having an I.H. of less than 0.2. A comparison of the chemical and immunochemical data yielded further information as to the nature of the carrageenan-anti-carrageenan interaction as well as elucidating the finer structure of these carrageenans.

5.) Carrageenans from female and male gametophytic plants of the alga *Rhodoglossum californicum*, female plants of *Chondrus crispus* and *Gigartina pistillata* and male plants of *Iridaea cordata* and a *Gigartina* species from San Francisco Bay were fractionated into KCl soluble and insoluble components and were analysed chemically. An anti-κ-carrageenan (G9-γ-477), the reactivity of which is directed to κ-type structures (i.e. 3-linked gal-4-SO_4 and 4-linked 3,6 AG) was used to analyse these carrageenans immunochemically. The KCl insoluble carrageenans from these species were found to be highly reactive κ-type carrageenans. The KCl soluble carrageenans were less reactive to anti-κ-carrageenan and in addition

showed reactivity to an anti-λ-carrageenan preparation. The chemical and immunochemical data suggest that the KCl soluble carrageenans contain either λ or ν carrageenan since a high proportion of the precursors to the 3,6 anhydride are 4-linked gal-2,6-di-SO_4 and no increase in immunological reactivity to anti-κ-carrageenan was observed upon alkaline treatment. The increase in 3,6 anhydride on alkali treatment of the KCl soluble carrageenans is not translated to an increase in κ-type molecules, synthesis of κ-carrageenan in the *Gigartinaceae*, therefore, may not be mediated, simply, through the enzymatic removal of 6-SO_4 from μ carrageenan; removal of 2-SO_4 on the 4-linked residue may be a necessary step in the synthesis of κ-carrageenan; (i.e. ν → ι → κ-carrageenan).

6.) An antibody preparation directed against a structural feature associated with 6-SO_4 (G7-γ-477) was used to probe the structural relations among certain λ-type carrageeans. Immunochemical and chemical differences were observed between the KCl soluble carrageenans from tetrasporic plants of *G. corymbifera, Gigartina* species from San Francisco Bay, *P. middendorfii, I. cordata, R. californicum* and *C. crispus*. The differences in immunochemical reactivity of the *Gigartina* and *Petrocelis* carrageenans relative to the homologous antigen are attributed to the lower content of 6-SO_4 on the 4-linked

residue in the former carrageenans; also, glc indicates the presence of the acid ketal 4,6-O-(1-CE)-D-gal which may contribute to the lowered immunochemical reactivity. Both the chemical and immunochemical data suggest that the *Gigartina* carrageenans are ξ-like in structure but do contain some λ-like features. The infrared spectrum of the *Petrocelis* carrageenan contains an unusually high content of pyruvated galactose. The carrageenan from *I. cordata* and *R. californicum* differ to a lesser degree from the *C. crispus* λ-type carrageenan. These differences cannot be accounted for by lower levels of 6-SO_4. Some other structural feature, as yet unidentified, is responsible for the discrepancy in the immunochemical reactivity of these carrageenans to the anti-λ-carrageenan.

7.) Combined glc/ms was used to seperate and identify a carrageenan component suspected of being the acid ketal 4,6-O-(1-CE)-D-gal. The mass spectral fragmentation pattern provides definitive proof for the presence of this component in the carrageenan from *P. middendorfii* (*franciscana*). This carrageenan represents a unique carrageenan polymer herein referred to as π-carrageenan, the idealized structure of which consists of alternating residues of α-1,3 linked 4,6-O-(1-CE)-D-gal-2-SO_4 and β-1,4 linked gal-2-SO_4.

MATERIALS AND METHODS

Carrageenan Extraction

Karyologically identified, desoried, freshly blotted algal samples were frozen in liquid nitrogen, pulverized in a ball mill, and the powder was extracted with several changes of acetone, hot 80% ethanol, 95% ethanol and diethyl ether. All samples were held overnight in vacuo over P_2O_5 before being weighed. Generally 0.2 to 0.4 g portions of this defatted dry powder were extracted with 0.5 M $NaHCO_3$ at 90° with vigourous stirring. The viscous liquid was recovered by pressure filtration through a thick glass-wool batt on top of a 12μ Sartorius membrane filter. Two re-extractions completed the removal of carrageenan from the insoluble debris which was retained on the glass-wool. The combined colorless carrageenan solutions (ca 200 ml) were refined through a series of stacked 12μ, 3μ and 1.2μ Sartorius membrane filters each separated by Whatman no. 1 filter paper. The carrageenan is precipitated with 0.2 - 0.4 g of Cetavlon (cetyltrimethylammonium bromide) dissolved in water. The Cetavlon was removed from the precipitated carrageenan complex by washing twice with distilled water and six times with saturated sodium acetate in ethanol. The sodium acetate was removed with warm ethanol (80%) and the whole carrageenan was dried and weighed as

described for the defatted powder.

Carrageenan Fractionation

Whole carrageenan was fractionated with KCl by a modification of the leaching proceedure of Stancioff and Stanley[3]; 200 mg of whole carrageenan were stirred into 50 ml of 0.3 M KCl and stirred overnight. The carrageenan solution was filtered with celite through a 3μ filter. The celite and filter were washed 2X with 15 ml 0.3 M KCl. The combined filtrate was passed through a 1.2μ Sartorius membrane filter under pressure. The soluble carrageenan in the filtrate was precipitated with 2½ volumes of 2-propanol and collected by filtration through a sintered glass filter. The precipitate was washed with 80% 2-propanol until the 2-propanol was free of chloride (tested with 1% silver nitrate), washed 2X with absolute 2-propanol and then with ethyl ether. The carrageenan was dried in vacuo overnight over P_2O_5. This fraction represents the KCl soluble carrageenan.

In order to recover the KCl insoluble carrageenan trapped by the celite, the filter cake was slurried with 25 ml of cold distilled water and heated to 85-90°. The carrageenan was filtered (while hot) through a Sartorius 3μ filter. The filter cake (celite) was washed 2X with 25 ml hot water. The filtrates were combined and diluted to 200 ml with hot distilled water. The carrageenan solution was filtered through

a 1.2μ filter while still hot. Enough 10% NaCl was added to make the carrageenan solution 1% NaCl and the carrageenan was precipitated with 2½ volumes of 2-propanol and collected in the same manner as the KCl soluble carrageenan.

Chemical Modifications

Preparatory alkaline borohydride modification was carried out according to a procedure described by Rees[46]. Typically 100 mg carrageenan were disolved in 25 ml of distilled water and 50 mg $NaBH_4$ was added. After 20 h at room temperature 3 N sodium hydroxide (12.5 ml) and 150 mg $NaBH_4$ were added; the mixture was heated at 80° for 7 h. The solution was transferred to a dialysis sac and dialysed with distilled water (10 l) for 24 h. The carrageenan was recovered by precipitation with 2½ volumes ethanol and collected on a sintered glass filter. The carrageenan was washed with 80% 2-propanol until free of salt, ethyl ether and dried in vacuo over P_2O_5 overnight.

Controlled alkaline borohydride modification was carried out by the same procedure except that samples were removed at various times from 15 min to 10 h and immediately precipitated with 2 volumes of absolute isopropanol. The modified carrageenan was collected by centrifugation and washed with 80% isopropanol until the samples were salt free. The samples were then dried as described above.

Periodate oxidation was carried out as described by Rees[46]. An 0.3% solution of carrageenan in 0.05 M sodium periodate was left at room temperature, in the dark, for 72 h. Ethylene glycol was added to destroy the excess of periodate and the solution analysed as usual for precursor units to the 3.6 anhydride (e.g. see below).

Gal-6-SO_4 and gal-2,6-di-SO_4 were determined as described by Lawson *et al.*[49], by determination of the increase in 3,6 anhydride on treatment with hot alkaline $NaBH_4$, before and after exposure to 0.05 M periodate for 72 h. The increase in 3,6 anhydride arises from the elimination of a equimolar amount 6-SO_4. Periodate oxidizable residues are gal-6-SO_4 while the periodate resistant residues are gal-2,6-di-SO_4.

3,6 Anhydride Content

The 3,6 anhydride content of carrageenans was determined by the method of Yaphe and Arsenault[48]. In general 100 ug of carrageenan and various amounts of the sucrose standard solution (43 ug/ml) were made up to a volume of 2 ml with distilled water. The samples were chilled on ice for 10 min. and 10 ml of resorcinol reagent, prepared by adding 1 ml (2.78 umoles/ml) acetal solution to 9 ml resorcinol solution (1.5 mg/ml H_2O) and adding this to 100 ml of concentrated HCl, were added to each sample. The samples were allowed to cool at least 3 min and not more than 30 min at 0°. The samples

were sealed and placed in a 20° water bath for 4 min, heated for 10 min in an 80° water bath (in the dark), cooled for 1.5 min in an ice bath and read at 555 nm within 15 min in diffuse light using an SP 1800 Unicam spectrophotometer.

Sulphate Determination

Sulphate was determined by the method of Jones and Letham[47]; 4 to 10 mg of carrageenan are dissolved in 1 ml 1 N HCl in an ampoule which is sealed with glass blowing equipment. The carrageenan is hydrolysed for 1 to 2 h in an oven at 105°. The tubes are allowed to cool, opened and the acid digest transferred to 10 ml volumetric flasks and diluted to 10 ml with H_2O. To 0.5 ml of the diluted sample a drop of 1% cetavlon, 0.5 ml 0.19% 4-amino-4'chlorodiphenyl in 0.1 N HCl is added. The samples are mixed by inversion and allowed to precipitate for 2 h, centrifuged and a 0.1 ml aliquot diluted to 10 ml in a volumetric with 0.1 N HCl. The solution is mixed by inversion and read in a Unicam spectrophotometer with an H_2 lamp at 254 nm; 0.01 N H_2SO_4 is used as a standard.

All chemical determinations were repeated 2X and performed in duplicate.

Infrared Spectroscopy

Infrared spectra were recorded for all carrageenan

fractions with a Perkin-Elmer model 283 spectrometer. Films of carrageenan were prepared by dissolving 2-3 mg of the material in a few drops of boiling distilled water and evaporating the solution on 25 mm diameter silver chloride windows at about 50°.

Gas-Liquid Chromatography

Carrageenan (1 mg) was submitted to methanolysis with 1 M HCl in anhydrous methanol (1 ml) for 20 h at 80° in sealed sialylized tubes. The methanolic HCl was evaporated under a stream of N_2 gas and trifluoroacetate (TFA) derivatives of the O-methyl-glycosides were prpared by reacting the methanolysate with excess (0.2 ml) TFA for 1 h at 80°. The TFA derivatives were analysed by glc on a 180 x 0.7 cm o.d. glass column containing 3% OV-210 Chromosorb W (80/100 mesh). The column temperature gradient was 110° to 200° (2°/min); the FID detector was at 350°; N_2 carrier gas flow was 40 ml/min.

Gas-Liquid Chromatography-Mass Spectrometry

TFA derivatives of the O-methyl-glycosides from *P. middendorfii* were prepared as described above. The TFA derivatives were separated by gas chromatography on a 180 x 0.4 cm o.d. column containing 3% OV-17 Chromosorb W (80/100 mesh) at a column temperature of 150°, and fed directly into a mass spectrometer. The mass spectrum of the compound with

retention time corresponding to 4,6-O-(1-CE)-D-gal was recorded at an ion source temperature of 200°, ionizing potential of 70 eV and an accelerating potential of 3 kV.

Pyruvate Determination

Pyruvated was detected by the enzymatic procedure of Duckworth and Yaphe[40]. This technique as well as the use of the 2,4 dinitrophenylhydrazone technique[39] have the shortcomming of requiring hydrolysis of the pyruvic acid moiety prior to its quantitation. This requirement may lead to problems of reproducibility as the lability of the acid ketal 4,6-O-(1-CE)-D-gal to acid is not known.

Since the area beneath a particular proton nuclear magnetic resonance (PMR) signal is directly proportional to the number of protons from which the signal is derived and in order to avoid the requirement for hydrolysis we have adapted a PMR technique to the quantitation of pyruvate in carrageenans. Pyruvate content is, therefore, quantitated by PMR spectroscopy of the native polysaccharide in D_2O at 75° and 100 Hz, using 1 umole sodium acetate per 2 mg polysaccharide as an internal standard. The PMR spectra was recorded on a Brucker WH-90 instrument.

Immunization

Antisera to various carrageenans were raised in goats

by 4 injections administered at two week intervals of various carrageenans conjugated to methylated bovine serum albumin (mBSA). Methylated bovine serum albumin was prepared by dissolving 100 mg BSA in 10 ml absolute methanol and adding 84 ul 12 N HCl; the mixture was allowed to stand at room temperature in the dark for three days with occasional mixing. The mBSA precipitates from solution and was collected by centrifugation. The precipitate was washed 2X with methanol, suspended in water and neutralized with NaOH. To complex with antigen, equal weights of mBSA (in solution) and antigen were mixed. The first injection consisted of carrageenan-mBSA (20 mg carrageenan) in 0.1 M PBS pH 7.4 emulsified with an equal volume of incomplete Freund's adjuvant. Subsequent injections consisted of carrageenan-mBSA solutions (20 mg carrageenan), administered subcutaneously in several sites. Two weeks after the last injection the goats were anesthetized, exsanguinated and the serum prepared as described below.

Antibody Preparation

The serum was dialysed against 0.1 M phosphate (PO_4) buffer pH 5.2-5.4 at 5° for 24 h to remove the water insoluble euglobulin which precipitates non-specifically with carrageenan. The globulin fraction is obtained by the slow addition of

finely ground ammonium sulphate ($[NH_4]_2SO_4$) to 50% saturation at room temperature. The precipitate was collected by centrifugation and redissolved in ½ volume of PBS pH 7.4. The immune γG-globulin fraction was precipitated by the slow addition of $[NH_4]_2SO_4$ to 33% saturation at room temperature and collected by centrifugation. The γG-globulin was dissolved in the same volume of PBS, dialysed against 10 l of PBS, sterilized by millipore filtration and stored at -70°. Four antibody preparations were prepared and labeled according to the goat serum from which they were prepared and the month and year of preparation; an anti-λ-carrageenan G7-γ-477, therefore, means that the γ-globulin was prepared from goat number 7 on the 4th month of 1977; two anti-κ-carrageenan G9-γ-477 and G15-γ-777 and an anti-ι-carrageenan G14-γ-1276 were similarly prepared and labeled.

Antigen-Antibody Reactions

Antigen (100 ug/ml) was added to 1 ml 0.3% agarose solution in 0.01 M phosphate buffered saline (PBS) at pH 7.5 at 45° in Bausch and Lomb Spectronic 20 tubes. The tubes were placed in a 37° waterbath and buffer was added to bring the volume to 2.5 ml. To this mixture was added 0.5 ml of the γ-globulin. The tubes were mixed vigorously in a vortex stirrer and placed in a Unicam SP 1800 double beam spectro-

photometer equipped with a static beam splitting mirror which sends the light beam for the monochromator equally through the sample and reference cells, at 37°. Precisely one minute after the addition of the antibody the external recorder read-out was engaged and the kinetics of precipitation were monitored by continuous read out at 420 nm.

Each antibody preparation was titrated by reacting varying amounts of the homologous antigen to a constant amount of antibody in a total volume of 3 ml (0.1% agarose in 0.01 M PBS pH7.5 at 37°) reaction mixture.

For the analysis of cross-reacting antigens, equal amounts (by weight) of the homologous antigen or cross-reacting substances were reacted with a constant amount of antibody in a total volume of 3 ml agarose reaction mixture. Both the rates of turbidity production and the total turbidity were recorded. The amount of antigen to be used was determined from the plot of initial rate of precipitation vs antigen (homologous) concentration at a point where the rate of precipitation is most sensitive to changes in the concentration of antigen antibody binding sites (antigenic determinants) (e.g see Chapter I).

All immunochemical reactions were performed in duplicate and repeated at least 2X. The reaction between the homologous antigen and the antibody preparation was always included in a set of reactions. Any set of reactions which

were to be compared were always carried out with the same agarose and buffer preparations and analysed concurrently.

REFERENCES

1. Di Rosa, M. (1972). J. Pharm. Pharmac. 24, 89.

2. Smith, D.B., Cook, W.H. and Neal, J.L. (1954). Arch. Biochem. Biophys. 53, 192.

3. Stancioff, D.J. and Stanley, N.F. (1969). IN: Proc. Int. Seaweed Symp. 9, 595.

4. Mueller, G.P. and Rees, D.A. (1968). Trans. Drugs from the Sea Symp., 241.

5. Penman, A. and Rees, D.A. (1973). J. Chem. Soc., 2182.

6. Anderson, N.S., Dolan, T.C.S., Lawson, C.J., Penman, A. and Rees, D.A. (1968). Carbohyd. Res. 7, 468.

7. DiNinno, V.L., McCandless, E.L. and Bell, R.A. (1978). Carbohyd. Res. (In Press).

8. Percival, E. and McDowell, R.H. (1967). Chemistry and Enzymology of Marine Algal Polysaccharids. Academic Press, London. 219.

9. Lawson, C.J. and Rees, D.A. (1970). Nature 227, 392.

10. Rees, D.A., Steele, I.W. and Williamson, F.B. (1969). J. polymer Sci., Part C, Polymer Symp. 28, 261.

11. Rees, D.A. Adv. Carbohyd. Chem. Biochem. 24, 267.

12. Johnston, K.H. and McCandless E.L. (1969). J. Immunol. 101, 556.

13. Mizushima, J., Murata, J. and Horiochi, Y. (1974). Int. Arch. Allergy 47, 532.

14. Sawichi, J.E. and Catanzaro, P.J. (1975). Int. Arch. Allergy appl. Immunol. 49, 709

15. Spector, W.G. and Ryan, G.B. (1969). Nature 221, 860.

16. Heidelberger, M. and Kendall, F.E. (1935). J. Exp. Med. 61, 559.

17. Libby, R.L. (1938). J. Immunol. 34, 269.

18. Pope. G.G. and Healey, M. (1938). Brit. J. Exp. Path. 19, 397.

19. Martin, D.S. (1943). J. Lab. Clin. Med. 28, 870.

20. Boyden, A., Bolton, E. and Gremory, D. (1947). J. Immunol. 57, 211.

21. Hosford, S.P.C. and McCandless, E.L. (1975). Can. J. Bot. 53, 2835.

22. Klotz, I.M. IN: The Proteins, Neurath, H. and Bailey, K. eds. (1973). Academic Press, N.Y. 1, 727.

23. Bernfeld, P., Donahue, V.M. and Berkowitz, M.E. (1957). J. Biol. Chem. 226, 51.

24. Painter, R.H. (1977). J. Immunol. 119, 2203.

25. Pinteric, S., Assimeh, S.N., Kells, D.I.C. and Painter, R.H. (1976). J. Immunol. 117, 79.

26. Doty, M.S. and Santos, G.A. (1978). Aquatic Bot. (In Press).

27. McCandless, E.L., Craigie, J.S. and Walter, J.A. (1973). Planta 112, 210.

28. DiNinno, V.L. and McCandless E.L. (1978). Immunochem. 15, 273.

29. Yaphe, W. (1973). Suppl., Proc. Nov. Scotia Inst. Sci. 27, 103.

30 DiNinno, V. and McCandless, E.L. (1977). J. Immunol. Meth. 17, 73.

31. DiNinno, V. and McCandless, E.L. (1978). Carbohyd. Res. (In Press).

32. DiNinno, V. and McCandless, E.L. (1978). Carbohyd. Res. (In Press).

33. Goodman, J.W. and Kabat, E.A. (1960). J. Immunol. 84, 333.

34. Evelegh, M.J., Vollmer C.M. and McCandless E.L. (1978). J. Phycol. 14, 89.

35. McCandless, E.L., Evelegh, M.J., Vollmer, C.M. and DiNinno, V.L. (1978). IN: Proc. Int. Seaweed Symp., 9 (In Press).

36. Heidelberger, M., Dudman, W.F. and Nimmich, W. (1970). J. Immunol. 104, 1321.

37. Araki, C. (1965). IN: Proc. Int. Seaweed Symp. 5, 3.

38. Hirase, S. and Watanabe, K. (1972). Bull Inst. Chem. Res. Kyoto Univ. 50, 30.

39. Sloneker, J.H. and Orentas, D.G. (1962). Nature 197, 478.

40. Duckworth, M. and Yaphe, W. (1970). Chem. and Ind. 747.

41. Morris, E.R., Rees, D.A., Young, O., Walkinshaw, M.D. and Darke, A. (1977). J. Mol. Biol. 220, 1.

42. Hirase, S. (1957). Bull Chem. Soc. Japan 30: 68,70 & 75.

43. Radford, T., DeJongh, D.C. IN: Biochemical Applications of Mass Spectrometry. Waller, G.R. ed. Wiley, N.Y. (1972). 313.

44. Polanshek, A.R. and West, J.A. (1975). J. Phycol. 2, 434.

45. Duckworth, M. and Yaphe, W. (1971). Carbohyd. Res. 16: 189, 435.

46. Rees, D.A. (1963). J. Chem Soc. 1821.

47. Jones, A.S. and Letham, D.S. (1965). Chem. and Ind. 662.

48. Yaphe, W. and Arsenault, G.P. (1963). Analyt. Biochem. 13, 143.

49. Lawson, C.J., Rees, D.A., Stancioff, D.J. and Stanley, N.F. (1973). J. Chem Soc. 2177.

50. Craigie, J.S. and Leigh, C. (1978). Carrageenans and Agar. IN: Handbook of Phycological Methods. J.A. Hellebust and J.S. Craigie, eds. Cambridge Univ. Press, Cambridge, Vol. 2 (In Press).

APPENDIX I

SOURCE OF CARRAGEENANS

CHAPTER I

Algal species	Stage of life cycle	Source	Details
C. crispus	carposporic	Dr. J.S. Craigie Atlantic Regional Lab. N.R.C.C. Halifax. N.S.	r: 9/3/76 e: VLD 29-7-76 † Fink Cove
C. crispus	tetrasposric	Dr. F. Loewus Dept. of Ag. Chem. Wash. State U. Washington	r: July/74 e: CV tet. 4 † Woods Hole Mass.
F. fastigiata	vegetative	Mr. J. Christiansen Litex Ltd. Glostrup, Denmark.	1/11/74 (A 949)
E. spinosum	*iota*-6060	Marine Colloids Inc. Rockland, Maine.	26/6/70
E. nudum	vegetative	Dr. C. Dawes Dept. of Biology U. of S. Fla. Tampa, Fla.	c: 9/9/74 e: C. Dawes alkaline mod. † Andote Buoy. Fla.

r: received
c: collected
e: extracted
†: collected at
CV: C. Vollmer

CHAPTER III

Algal species	Stage of life cycle	Source	Details
C. crispus	←←←←←←←←←← As in Chapter I →→→→→→→→→→→→→→→→→→→→→→→→		
E. cottonii	female (#3)	Dr. M. Doty Dept. of Botany U. of Hawaii Honolulu, Hawaii.	s: 12/4/77 MD 24975 e: CV
E. cottonii	tetrasporic (#2)	Dr. M. Doty "	"
E. serra	female (#4)	Dr. M. Doty "	s: 12/4/77 MD 24706 e: CV
E. serra	tetrasporic (4)	Dr. M. Doty "	"
E. nudum	female (non-sporulating)	Dr. C. Dawes	c: 11/11/74 e: CV † Vero Beach Fla.
E. nudum	tetrasporic	Dr. C. Dawes	"
E. striatum (var Tambalang)	female	Dr. G. Santos Dept. of Botany U. of Hawaii Honolulu, Hawaii	G. Santos # 31505 e: CV
E. isiforme	R.E. 7391	Mr. D.J. Stancioff Marine Colloids Inc.	27/1/76
E. spinosum	R.E. 7389	"	"
A. tenera	R.E. 7390	"	"

c: collected
e: extracted
†: collected at
s: shipped
CV: C. Vollmer

CHAPTER IV

Algal species	Stage of life cycle	Source	Details
C. crispus	←←←←←←←←←←←←←←←←←← As in Chapter I →→→→→→→→→→→→→→→→→→		
I. cordata	female	Mrs. J. Hansen Div. of Natural Sci. U. of Cal. Santa Cruz, Cal.	c: 16/10/74 e: CV
R. californicum	female	Mrs. J. Hansen "	c: 29/3/75 e: CV
R. californicum	male	Mrs. J. Hansen "	c: 6/3/75 e: CV † Ano Nuevo Pt.
G. pistillata	female	Dr. J. Cabioch Station Biologique 29211 Roscoff, Finistière, Fr.	s: 9/75 # 372
Gigartina sp. (S.F.B)*	female (#10)	Mr. A. Nonamura Botany Dept. U. of Cal. Berkeley, Cal.	c: 6/8/75 † Shorline Dr. Alamedia Cal.

c: collected
s: shipped
†: collected at
* San Francisco Bay
e: extracted
CV: C. Vollmer

CHAPTER V

Algal species	Stage of life cycle	Source	Details
C. crispus	tetrasporic	←←←←←← As in Chapter I →→→→→→	
C. crispus	REX 5969	Marine Colloids Rockland Me.	r: 13/2/70 e: 5937
R. californicum	tetrasporic	Mrs. J. Hansen	c: 29/3/75 e: CV † Ano Nuevo Pt. Cal.
I. cordata	tetrasporic	Mrs. J. Hansen	I.c. #8 c: 11/9/74 I.c. # 12 c: 16/10/74 e: CV † Ano Nuevo Pt. Cal.
G. corymbifera	tetrasporic	Dr. J. Stein Dept. of Botany U.B.C. Vancouver, B.C.	G.c. #4 r: 25/7/73 e: CV † Vancouver
Gigartina sp. (S.F.B.)*	tetrasporic	Mr. A. Nonamura	G.f # 11 c: 6/8/75 e: CV † Almedia Cal.
P. middendorfii (*P. franciscana*)	tetrasporic	Dr. J. West Dept. of Botany Berkeley, Cal.	c: 25/10/73 e: CV † Mission Pt. Monterey Co., Cal.

r: received
c: collected
e: extracted
CV: C. Vollmer
* San Francisco Bay