STUDIES ON CARRAGEENAN

STUDIES ON INCORPORATION OF ^{14}C INTO CARRAGEENAN AND METHODS OF LOCALIZING CARRAGEENAN IN ANIMAL TISSUES

By

SUZANNE M. RICHER, B.Sc.

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

October, 1971

MASTER OF SCIENCE (1971) McMASTER UNIVERSITY
(Biology) Hamilton, Ontario

TITLE: Studies on incorporation of ^{14}C into carrageenan and methods of localizing carrageenan in animal tissues

AUTHOR: Suzanne M. Richer, B.Sc. (Marianopolis College)

SUPERVISOR: Professor E. L. McCandless

NUMBER OF PAGES: v, 99

SCOPE AND CONTENTS:

Lambda carrageenan when injected subcutaneously causes the formation of a connective tissue granuloma. Initially there is a proliferation of connective tissue elements up to about fourteen days followed by regression so that by six weeks most collagenous tissue has disappeared and been replaced by adipose tissue. Lambda carrageenan has been identified in the granuloma by staining reactions with toluidine blue and other stains for acid polysaccharides. The present study was undertaken to localize the carrageenan by means of fluorescent antibody and autoradiography. For this purpose labelling of carrageenan by photoassimilation of $^{14}CO_2$ into carrageenan was done. Different parameters affecting the incorporation of ^{14}C into the carrageenan fractions were studied.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. E. L. McCandless for her supervision, encouragement and generous assistance throughout this study. The author is grateful to Dr. E. M. Gordon and Mr. K. H. Johnston for their assistance in the preparation of this thesis. I wish to thank Miss B. Hastings and Miss C. Whittington for their technical assistance. Special thanks are due to Dr. J. L. McLachlan for the generous supply of *Chondrus crispus* from Nova Scotia and to Marine Colloids, Inc. for the supply of lambda carrageenan.

The financial assistance of an Ontario Graduate Fellowship is acknowledged.

TABLE OF CONTENTS

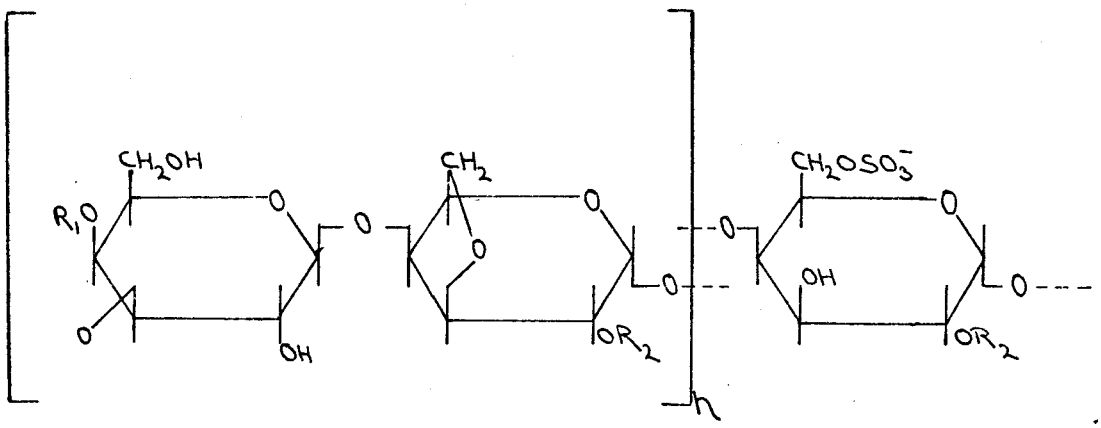
INTRODUCTION

A. Structure of carrageenans

The carrageenans are sulphated polysaccharides found in some species of the Rhodophyceae. The name carrageenan was originally applied to the polysaccharide from the genera Chondrus and Gigartina but compounds of similar structure are found in plants of the genera Furcellaria, Eucheuma, Hypnea, Iridaea, and Polyides (Percival and McDowell, 1967). The carrageenans are galactans which can be extracted from the plant with hot water or dilute alkali, then recovered by precipitation with alcohol. Smith and Cook (1953) separated carrageenan into two components by fractional precipitation with potassium chloride (0.25M KCl) and designated the fraction precipitated in the presence of potassium ion as K-carrageenan and that remaining in solution as λ-carrageenan. Since fractionation is sharp, Smith and Cook (1953) postulated the presence of at least two distinct fractions and not just a separation of particles heterogeneous in size. Lambda carrageenan has a higher molecular weight (330,000 to 790,000) than K-carrageenan (260,000 to 320,000)(Smith et al, 1954).

Kappa carrageenan consists of an alternating chain of 1,3-linked D-galactose and 1,4-linked 3,6-anhydro-D-galactose (O'Neill, 1955). The degree and position of sulphation varies with the species or genera as well as the preparation used. Methylation studies (Dolan, 1965) and infrared spectroscopy (Anderson et al, 1968) of K-carrageenan from Chondrus crispus have shown that galactose 4-sulphate is the only 3-linked unit but the sulphation of the 4-linked unit is to some degree variable.

An unsulphated 3,6-anhydrogalactose unit is the idealized representation and a literal one for a proportion of molecules in a preparation. However; some 4-linked 3,6-anhydrogalactose 2-sulphate, galactose 6-sulphate, and galactose 2,6-disulphate are present in K-carrageenan. The structure proposed for K-carrageenan is as follows:



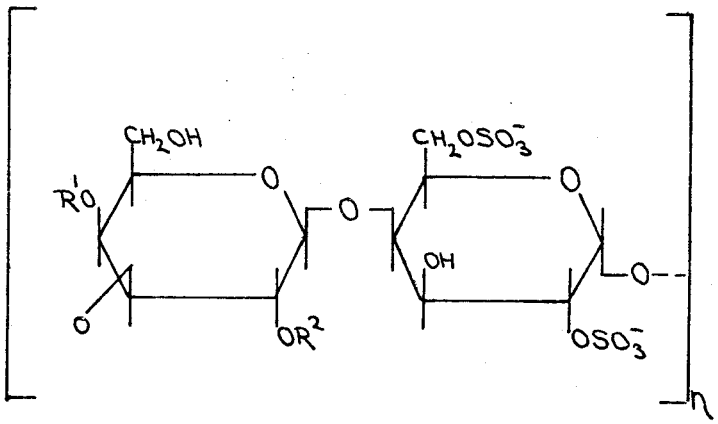
$R_1 = SO_3$

R_2 usually H, can be SO_3^-

The presence of 1,4-linked galactose 6-sulphate is confirmed by a loss of sulphate with a concomitant increase in 3,6-anhydrogalactose upon alkali treatment of K-carrageenan. This was further confirmed by enzymatic studies with K-carrageenase (Weigl et al, 1966). Eighty percent of the K-carrageenan was degraded by the enzyme, the remaining 20% was rendered degradable after alkali modification. These results are also supported by paper chromatographic identification of galactose 2-, 4-, and 6-sulphates after partial acid hydrolysis of unmodified K-carrageenan (Painter, 1966).

Lambda carrageenan was first considered to consist almost entirely of 1,3-linked D-galactose units. Rees (1963) showed that a 1,4-linkage

was also present in λ-carrageenan and that 40-50% of the structural units consisted of 4-linked galactose 2,6-disulphate. Dolan and Rees (1965) proposed the following model for λ-carrageenan in which equal proportions of 1,3-linked and 1,4-linked units are present:



(I) R^2 usually SO_3^-, can be H

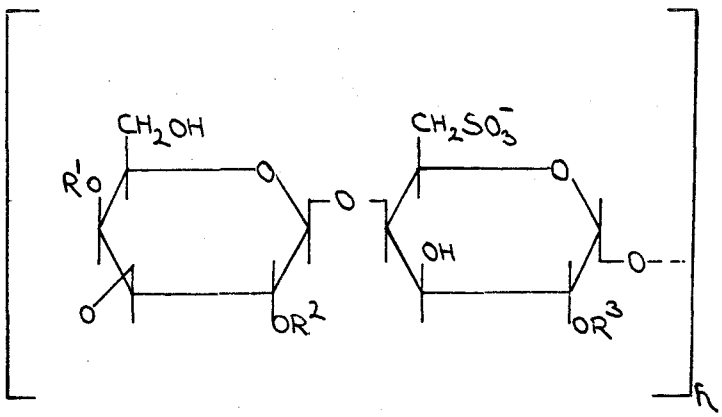
when R^2 = H R^1 usually H, can be SO_3^-

(II) R^2 varies from unit to unit but is usually SO_3^- R^1= H.

The composition of the fraction soluble in KCl and defined as λ-carrageenan has shown considerable variability from sample to sample. Contamination with 3,6-anhydrogalactose has been frequently found. Experimental evidence has been interpreted to support structure (I), above. Because the variability in the fraction is considered to be due in most part to contamination with other carrageenan components, Dolan and Rees (1965) propose to define ideal λ-carrageenan as a molecule devoid of 4-sulphate and 3,6-anhydrogalactose. This is structure (II) in the preceding diagram. Thus the definition of the carrageenans is now based on structure rather than on solubility properties.

The existence of another component in the KCl soluble fraction was

first shown by Dolan and Rees (1965). This "third component" accounts for the variability in the KCl soluble fraction. The "third component" is designated mu (μ) carrageenan (Anderson, et al, 1968). The difference between λ- and μ-carrageenan lies in the distribution of sulphate ester groups. The idealized structure of μ-carrageenan is as follows:



$R^1 = SO_3^-$, $R^2 = R^3 = H$ (some $R^3 = SO_3^-$)

Thus, Chondrus crispus is now considered to have three carrageenan components: K, λ, and μ. Because of the extensive rearrangement of sulphate groups required to make K- from λ-carrageenan Rees (1965) suggests that K- and λ-carrageenan are (relative) end-products of biosynthesis which might, however, undergo metabolic alteration in vivo to a small extent. The structure of μ-carrageenan suggests that it may be the precursor of K-carrageenan.

B. Carrageenan effect

i) Connective tissue response

Carrageenan when injected subcutaneously induces the formation of a connective tissue granuloma. This observation was first made by Robertson and Schwartz (1953) in a study of the effect of ascorbic acid on collagen formation. Robertson and Schwartz (1953) give a microscopic description of the effect of an injection of an extract of Chondrus crispus

into the abdominal subcutaneous tissue of guinea pigs. The swelling at the site of injection had disappeared by twenty-four hours. After five days, a gel-like mass which oozed a slightly viscid fluid was found at the site of injection. Later a contraction and firming of the tissue occurred so that by fourteen days a dense fibrous tissue was found. After three weeks a slow resorption of the tissue was noted and by six weeks only a small amount of firm fibrous tissue remained. The major portion of collagen was laid down between the tenth and fourteenth days. Williams (1957) did the first histological study of the carrageenan granuloma in guinea pigs. By the third day after injection a semi-gelatinous and partly haemorrhagic layer of tissue was present, containing polymorphonuclear leucocytes and monocytes with "ingested" carrageenan identified by metachromatic staining and a positive periodic acid-Schiff reaction. By the fifth day the granulation tissue had been invaded from the overlying dermis and underlying muscle by capillaries and fibroblasts. The presence of histocytes with ingested metachromatic carrageenan was noted. The surrounding ground substance was also metachromatic but this metachromasia was hyaluronidase sensitive whereas that in the histocytes was not. By the tenth day much of the "extracellular" carrageenan had disappeared and the tissue was now highly cellular, containing histocytes and fibroblasts as well as a fine reticulum network. Fibrosis proceeded from the peripheral zone towards the center of the granuloma. By fourteen days, mature collagen was present and resorption of the first formed collagen fibers had begun. The resorption was complete by six weeks leaving only areas of loose adipose tissue. A loss of collagen in the dermis was noted. Injection of carrageenan into the dermis was found to

cause a permanent loss of its collagenous tissue. Jackson (1956 a and b, 1957) found an increase in collagen precursor fractions in the early granuloma and in insoluble collagen to fourteen days, followed by increased soluble collagen due to breakdown of the insoluble collagen. Slack (1957) measured the sulphate content of the carrageenan granuloma and found an increase to about six days indicating a rapid synthesis of polysaccharide; then a decline to normal levels by the fourteenth day. Williams (1957) found a decrease in the metachromasia of the ground substance as the granuloma became more fibrous.

All the previous studies were done using unfractionated extracts. McCandless and Lehoczky-Mona (1964) demonstrated that lambda carrageenan was the fraction responsible for the connective tissue response and minimal if any activity was attributed to kappa carrageenan. The studies of McCandless and Lehoczky-Mona (1964, 1966) and McCandless (1965) indicate that for a compound to have carrageenan-like connective tissue activity it must be a linear galactose polymer with a 1,3 or an alternating 1,3 and 1,4-linkage; sulphation was not an absolute requirement. Injection of lambda carrageenan fragments of differing molecular size showed that a certain degree of polymerization was necessary for activity.

Carrageenan granulomas have been studies in species other than guinea pigs. Granulation was observed in rats (Benitz-Hall, 1959; Fisher and Paar, 1960; Morris et al, 1968; Stassen, 1970) and in rabbits (Pérez-Tamayo, 1970). The granulomatous response in rats is similar to that in guinea pigs but is of a more cellular nature and of much longer duration. Stassen (1970) observed rat granulomas still present at 100 days, although there was some replacement by adipose

tissue at this time.

Several explanations for the connective tissue response have been postulated. Williams (1957) suggests that the carrageenan granuloma is a foreign body response since carrageenan is a large molecule with a slow diffusion rate and the granuloma is a result of the organism's attempts to remove it. McCandless (1965) suggests that due to the similarity of carrageenan to chondroitin sulphate it may substitute for the naturally occurring mucopolysaccharide during granuloma formation.

ii) Antibody stimulation

A chance observation by McCandless (1967) that a second injection of carrageenan caused an exaggerated tissue response led to the discovery of the antigenicity of carrageenan by Johnston and McCandless (1967). They showed the presence of precipitins to lambda and kappa carrageenan in serum obtained from rabbits which had been injected intravenously with the purified carrageenan fractions. The carrageenans were administered as complete antigens without the use of adjuvant (Johnston and McCandless, 1968). Antibodies produced to lambda carrageenan did not precipitate kappa carrageenan and likewise anti-kappa serum failed to precipitate with lambda carrageenan. Thus these antibodies could be used as a specific assay for the carrageenan fractions.

C. Problem

As suggested by Pérez-Tamayo (1970), there is no direct proof for localization of carrageenan in the granuloma. A specific study of the fate of both extracellular and intracellular carrageenan in the granuloma is not available. The criteria used for identification of carrageenan

have been its staining reactions with certain dyes, the main one being a metachromatic reaction with toluidine blue. The periodic acid-Schiff reaction has also been used for identification. Although these dyes react with carrageenan *in vitro* and presumably *in vivo*, they also react with other substances such as the mucopolysaccharides of connective tissue.

The present study was undertaken to obtain direct proof of carrageenan localization in the granuloma. Two main approaches to this problem were used. The first approach was by autoradiography of sections of granuloma induced by the injection of ^{14}C labeled carrageenan. Hellebust and Haug (1969) obtained ^{14}C labeled alginic acid by allowing *Laminaria digitata* to photoassimilate $^{14}CO_2$. Similar photoassimilation experiments with *Chondrus crispus* were used to obtain ^{14}C labeled carrageenan. In the course of these experiments the amount of label appearing in the different carrageenan fractions and the effect of altering parameters such as light conditions of incubation became interesting *per se*, and form a separate part of the thesis.

The second approach to localization depended on the antigenic behavior of the carrageenans. An attempt was made to identify lambda carrageenan in the granuloma by fluorescent antibody techniques. The only report in the literature of a fluorescent antibody reaction to algal polysaccharides described the complexing of antibody to alginic acid extracted from *Fucus distichus*, a brown alga (Vreeland, 1970, 1971). The specificity of an antibody for its homologous antigen suggested the use of fluorescent antibody techniques to localize carrageenan in the connective tissue granuloma.

CHAPTER II

MATERIAL AND METHODS

A. Carrageenans:

Lambda carrageenan is defined as that alcohol precipitable portion of an aqueous extract of the appropriate algal source which is soluble in KCl. Kappa carrageenan is the KCl insoluble fraction. The concentration of KCl which defines kappa carrageenan, however, depends on the specific fractionation procedure and tends to be an arbitrary decision. For the production of antibody, lambda carrageenan prepared by Marine Colloids, Inc., Rockland, Maine, was used. The analysis of this lambda carrageenan was provided by Marine Colloids:

Ester SO_4^- 36.7%

3,6-Anhydrogalactose 2-3%

viscosity (1.5% solution, 75°C) 888 cps

This lambda carrageenan was used in all studies except ^{14}C labeled carrageenan experiments. The extraction, fractionation and analysis of carrageenans in the ^{14}C labeling experiments will be described in the appropriate section of material and methods.

B. Chondrus crispus

Chondrus crispus Stackhouse belongs to the class Rhodophyceae (red algae). Chondrus crispus grows on rocks near low tide level or in tide pools, carpet-like or sheltered by larger brown algae. Occasionally, it has been observed in nature existing planktonically (Fritch, 1945) and also grows well in this state in artificial seawater (Instant Ocean-Aquarium Systems, Inc.) in an aquarium. The thallus is 8-15 cm. tall

and each plant is composed of a small disk-like holdfast giving rise to one or more erect fronds which are more or less dichotomously divided, the stipe and segments compressed and 2-15 mm. broad, with rounded apices. The tetrasporangial and cystocarpic sori occur near the apices of mature fronds.

C. Labeling of carrageenan with ^{14}C

1. Photoassimilation of ^{14}C bicarbonate

The method adopted for the incorporation of ^{14}C into carrageenan was essentially that of Hellebust and Haug (1968). The seaweed, after shipment from Nova Scotia, was maintained in artificial seawater (Instant Ocean-Aquariums Systems, Inc.) in aquariums kept at 8-15°C. In this text, the terms Instant Ocean and seawater are used interchangeably. In no instance was actual seawater used. All fronds selected for the experiments were washed in fresh seawater, blotted dry, weighed; large fronds were cut to permit total immersion, and placed in a plastic container of Instant Ocean to which had been added the $NaH^{14}CO_3$. The temperature was kept between 4 and 8°C by placing the plastic container on an ice bath. A fluorescent F15T12-CW Sylvania light was placed over the seaweed and the light intensity regulated by adjusting the position of the light. The fronds were arranged so that each one received direct illumination.

A total of four ^{14}C incorporations were done on seaweed collected at various times of the year and subjected to different conditions. The first (experiment #1) used Chondrus crispus collected off the coast of Nova Scotia near Halifax. The seaweed was packaged with ice and sent air express to our laboratory where it was kept in an aquarium until use. The seaweed was collected June 8, 1969 and the labeling experiment was

done on July 7, 1969. About 50 g of seaweed were placed in 500 ml of Instant Ocean which contained 100 μc of $NaH^{14}CO_3$ (20 μc/100 ml seawater). The light was adjusted to deliver about 250 foot candles (2310 lux) at the level of the seaweed. The fronds were exposed to continuous light for the duration of the experiment. Samples were removed at 12, 24, and 48 hours, rinsed in fresh seawater and dried.

For experiment #2, the Chondrus crispus was collected off the coast of Nova Scotia, near Halifax on September 4, 1969. The seaweed was sent air express as before and maintained in an Instant Ocean aquarium in a cold room. The seaweed was maintained on a 12 hour light/dark cycle during this time. The incorporation experiment was begun on September 9, 1969. Two plastic containers were used, each containing about 50 g of seaweed and 200 μc $NaH^{14}CO_3$ (specific activity 4.6 mc/mM) in 500 ml Instant Ocean (40 μc/100 ml seawater). The light was set to deliver 250 foot candles as before and the experiment done in continuous light. After 93 hours, the radioactive seawater was removed and replaced with non-radioactive seawater for three additional hours in continuous light. The seaweed was then rinsed twice in seawater and allowed to dry.

Experiment #3 used Chondrus crispus collected at Fink Cove, Nova Scotia on January 26, 1970 and maintained in a cold room on a 12 hour light/dark cycle until February 16, 1970 when the experiment was started. Only non-sporulating fronds were used in this case. Thirty-six grams of seaweed were placed in 300 ml Instant Ocean containing 0.3 ml $NaH^{14}CO_3$ (100 μc/100 ml seawater). The specific activity of the bicarbonate was 11.4 mc/m M. The light intensity was 160 foot candles (1440 lux). The experiment was carried out with a 12 hour light/dark cycle beginning when

the seaweed was placed in the radioactive seawater. Samples were taken at 6, 10, 24, 34, and 48 hours; each was given a two hour chase in non-radioactive seawater. Two samples were taken at 48 hours, one was given a two hour chase, the other a six hour chase. All chases were done in the light.

On February 18, 1970, experiment #4 was started using non-sporulating seaweed from the same source as in experiment #3. The conditions were the same as for experiment #3 as far as concentration of bicarbonate, amount of seaweed per 100 ml seawater and light intensity; however, the light was continuous rather than on a light/dark cycle. Samples were taken at 6, 10, 24, 34, and 48 hours and given a two hour chase in non-radioactive seawater, then rinsed in fresh seawater and dried. Three samples were taken at 48 hours, one was not chased but merely rinsed in fresh seawater, one given a two hour chase, and one given a six hour chase. All chases were done in the light.

The seaweed from experiment #1 and #2 were extracted and fractionated according to procedures a) described on pages 13 and 14 respectively. In addition, some carrageenan extracted in experiment #2 was fractionated at 0.3M KCl according to fractionation procedures a) and b). The fraction insoluble at 3M KCl in experiment #2 was refractionated at 0.25M KCl and at 0.125M KCl.

The seaweed in experiment #3 was extracted and fractionated according to procedure b). The kappa carrageenan fraction was subsequently refractionated according to the method of Smith, Cook, and Neal (1954) described on page 15.

2. Extraction procedures

a) For the initial experiments the extraction procedure was as follows:

Dried seaweed in 0.04N NaOH (10 ml NaOH/200 mg Chondrus crispus) was heated for 5 hours at 75°C. The partially digested seaweed was then macerated by grinding it through a tea strainer with a pestle and heating continued for one hour. The resulting viscous solution and debris were centrifuged at 2000 rpm, 40 minutes. The supernatant was precipitated with 3 volumes of 95% ethyl alcohol. The precipitate was of a strong jelly-like consistency and was easily collected on a glass rod. The precipitate was washed twice with 95% ethyl alcohol, twice with absolute alcohol and finally with ether, then allowed to dry.

b) To improve the percentage yield, a modified procedure was adopted for the later experiments. This procedure was provided by Mr. D. J. Stancioff of Marine Colloids, Inc. (personal communication). The dried weed was washed in distilled water to remove salt. The washed seaweed was heated in 0.04% NaOH (100 ml NaOH/2g seaweed) to 90°-95°C in a boiling water bath. It was then covered tightly and held overnight at room temperature. The mixture was then milled in a Waring Blender, at low speed, for one minute and heated in a boiling water bath an additional two hours. To aid filtration, 5g Celite 545 (Johns Manville)/100 ml solution was added and mixed for 30 minutes. The mixture was then filtered in a pressure filter. The filter cake was washed with hot water and washings added to the filtrate. Five ml 10% NaCl/100 ml filtrate were added and mixed well. The filtrate was cooled to 50-55°C and poured slowly into $2\frac{1}{2}$ volumes of 85% w/w isopropyl alcohol. After stirring, the coagulum was allowed to stand for 30 minutes, after which the alcohol

was drained off through cheesecloth and the remaining alcohol squeezed from the coagulum. The coagulum was picked apart and soaked in about 100 ml 85% isopropyl alcohol for one hour. Finally the alcohol was drained off and the coagulum allowed to dry.

3. Fractionation

a) In the initial experiments the fractionation was done in a 3M KCl solution. The potassium chloride was added either as a fine powder or as a slurry to a 0.1% solution in water of the extracted carrageenan. The solution was stirred for four hours at room temperature. The precipitated fraction was collected by centrifugation at 2000 rpm, 40 minutes. The precipitate was slurried in water and dialysed overnight against running tap water to remove KCl. The supernatant was also dialysed against running tap water overnight. The volume of the supernatant was reduced to 1/3 by heating at 60°C. Both the KCl soluble and KCl insoluble fractions were precipitated with three volumes of 95% ethyl alcohol and collected by centrifugation. The precipitates were washed twice with 95% ethyl alcohol, twice with absolute alcohol and finally with ether before the precipitates were dried.

b) A modification, provided by D. J. Stancioff, of the fractionation procedure was adopted in the later experiments to improve the yields and purity of the fractions. One gram of carrageenan/100 ml 2.5% KCl (0.3M) was stirred for one hour at room temperature. The mixture was held overnight. It was stirred again for one hour, 5 g of Celite was added, and the stirring continued for ten minutes. The mixture was filtered under pressure and the filter cake washed with 2.5% KCl. The filtrate was coagulated in 2½ volumes of 85% isopropyl alcohol for each

volume of filtrate. The coagulum was recovered by centrifugation (2000 rpm, 20 minutes). The coagulum was washed repeatedly in 85% isopropyl alcohol until there was no more chloride in the washings, as indicated by an $AgNO_3$ test. The coagulum (lambda carrageenan) was allowed to dry.

The filter cake was slurried in about 100 ml cold deionized water until all lumps were dispersed.

This was heated to 90-95°C in a boiling water bath and filtered while hot (the KCl insoluble fraction forms a gel when cool). The filter cake was washed with hot deionized water and the wash added to the filtrate. The filtrate was coagulated as above in 2½ volumes of isopropyl alcohol and washed free of chloride. The KCl soluble fraction is designated as lambda carrageenan and the KCl insoluble fraction as kappa carrageenan. Carrageenan fractionated by this method gives a very pure lambda fraction. The kappa fraction may contain a small amount of lambda and mu carrageenan.

A purer kappa carrageenan was obtained using the method of Smith, Cook, and Neal (1954) which essentially involves refractionation of the 0.3M KCl insoluble fraction at 0.15M KCl. It was considered that contamination by lambda and mu carrageenan would be reduced by this procedure as they should be KCl soluble. The 0.3M insoluble fraction was dissolved in water and 1M KCl added to give a final concentration of 0.15M in KCl, let stand overnight and the two fractions recovered by centrifugation or by filtration through cheesecloth.

4. Analysis of carrageenan

Certain chemical tests were performed on the extracted and fractionated products obtained from the labeling experiments.

Measurement of sugar was done by the phenol sulfuric method of Dubois et al (1956), using galactose as a standard. This method is considered to be accurate to ±2%. The content of 3,6-anhydrogalactose of the different fractions was measured by the modified resorcinol method as described by Yaphe and Arsenault (1965), using fructose as a standard; 3,6-anhydrogalactose has an absorbance equal to 92% that of fructose at 555 mμ. Reducing sugar was measured by the Somogyi (1952) modification of the method of Benedict.

Hydrolysis of carrageenan was performed by mixing equal volumes of a 0.5% carrageenan solution and 45% formic acid. The mixture was heated for 16 hours at 100°C to effect hydrolysis. The products were analysed by ascending paper chromatography on Whatman #1 filter paper. The hydrolysis products were identified by comparison with known sugars. The solvents used in chromatography were:

a) n-Butanol:ethanol:H_2O 3:1:1.
   plus 3% cetyl pyridinium bromide (Rees, 1960)

b) n-Butanol:ethanol:H_2O 3:1:1.

c) n-Butanol:pyridine:H_2O 6:4:1.

To identify the sugars the following sprays were used:

a) To test for reducing sugar, 3% anisidine hydrochloride in butanol (Pridham, 1956)

b) To test for acid polysaccharide, toluidine blue (Hamerman, 1955)

c) To test for reducing and non-reducing sugars, silver nitrate (Dolan, 1965)

The analysis for localization of radioactivity on the chromatogram was done by cutting out the spots and counting in scintillation fluid or

by cutting the chromatogram into equal portions from the origin to the solvent front, and counting in scintillation fluid.

D. Metabolism experiments

An experiment was designed to study the localization and excretion of carrageenan after its subcutaneous administration to guinea pigs. Immediately after subcutaneous injection of ^{14}C lambda carrageenan, each animal was placed in a wire cage constructed to collect urine in a pan, the feces being caught on a wire mesh above the pan. The entire cage was covered by a large bell jar which was sealed, with window putty, to the platform on which the cage stood. There was an intake tube for air entry into the bell jar and an outlet tube connected to an aspirator. The air from the bell jar was passed through five gas washing bottles of 1N NaOH connected in tandem to remove expired CO_2. Urine and expired CO_2 samples were taken and the cage cleaned every 24 hours. The guinea pigs were supplied with food and water at all times. In the longest experiment of this kind, the guinea pig was maintained in the cage for four days without any noticeable ill effects and without undergoing weight loss.

E. Autoradiography

Autoradiography was carried out on the parietal lymph node, spleen, and granuloma tissue of guinea pigs injected with ^{14}C lambda carrageenan. The tissues were fixed either in 10% neutral buffered formalin or by the procedure of Sainte-Marie (1962). After hydration, the slides were coated with NTB_2 Kodak nuclear emulsion. In a dark room, with a 15 watt safe light, the gelled emulsion was scooped into a Coplin jar, in a water bath at 40°C. When melted the emulsion was stirred, gently, to avoid introducing air bubbles. The slides were then dipped three times in a

period of five seconds into the emulsion (Gude, 1968). The slides were allowed to drain in a vertical position for 30 seconds, then placed in an exposure chamber of a Con/Rad Joftes Fluid Emulsion Radioautography System and exposed according to the instructions provided for the chamber. Essentially they are exposed at room temperature in a dry CO_2 atmosphere. This procedure was later modified in that the emulsion was diluted with water, one part water to two parts emulsion and the slides heated to 40°C before dipping to give a more uniform coating. Slides were also dry when dipped to give a uniform coat. To minimize background, some slides were dried one hour in a light proof box, then exposed to 3% hydrogen peroxide vapors for 3½ hours. This was done by placing the slides in staining jars lined with filter paper soaked in 3% H_2O_2. The slides were then removed and allowed to dry for 15 minutes (Prescott, 1964). These slides were exposed as usual in the Con/Rad Joftes exposure chamber. Control slides were included in all experiments and slides were removed at intervals to determine proper exposure times. The autoradiograms were developed according to the procedure in the Con/Rad Joftes Instruction Manual for the Fluid Emulsion Radioautography System. Kodak D19 developer was used for 1 to 10 minutes, stopbath 15-30 seconds, and acid hard fixer 1-5 minutes. The slides were then washed in running tap water and stained either with toluidine blue or with hematoxylin and eosin.

F. Fluorescent antibody

1. Immunization

For immunization, the lambda carrageenan was dissolved as a 1% solution in 0.9% NaCl phosphate buffered to pH 7.1. This solution was dialysed for 18 hours against phosphate buffered saline and autoclaved

for 10 minutes, 18 psi. The antigen was injected into the marginal ear vein of each of two rabbits which weighed about 2 kg each. A total of 35 mg carrageenan was injected at three day intervals over a three week period, following an injection schedule customarily used in this laboratory. The rabbits were maintained on a Purina chow diet, food and water were available at all times. Blood was taken from the central ear artery on the day of the first injection and on day four after final immunization. Two weeks after the last injection the rabbits were anesthetized and exsanguinated by heart puncture.

2. Isolation and assay of antibody

The blood was maintained at 37°C for two hours after bleeding, then stored at 4°C for 18 hours to permit clot formation and retraction. The serum was then decanted and centrifuged twice at 10,000 xg, 20 minutes at 4°C. The serum was preserved by adding 1% methiolate to a final concentration of 1:10,000 and stored at 4°C. To destroy complement, the serum was heated to 56°C for 45 minutes before precipitation of the γ-globulin was carried out.

The precipitation of the γ-globulin was performed according to the method of Weir (1967). All procedures were done at 4°C. One volume of saturated ammonium sulfate was added dropwise, with constant stirring, for every two volumes of serum. The mixture was allowed to stand overnight, then centrifuged at 8000 rpm, 30 minutes. The precipitate was washed twice with 40% saturated ammonium sulphate, then dissolved in one volume of phosphate buffered saline (0.1M pH 6.5). The γ-globulin was reprecipitated with one half volume saturated ammonium sulphate. The washing with 40% saturated ammonium sulphate and redissolving was repeated a total of three times. The final precipitate was stored in

40% saturated ammonium sulphate. The precipitated γ-globulin was dissolved in phosphate buffered saline (pH 6.5) and dialysed free of sulphate before use. $BaCl_2$ was used to test for sulphate in the dialysis solution. Precipitation tests were performed as follows on the pre-injection γ-globulin and on the γ-globulin of trial bleeding done four days after final injection. To 0.1 ml of γ-globulin solution was added 0.9 ml of antigen solution of various concentrations so that from 10 to 150 µg of antigen was added. After mixing, the tubes were incubated at 37°C for 2 hours then stored at 4°C for 7 days. The tubes were then centrifuged at 12,500 rpm at 4°C for 3 minutes, drained and washed with one ml of saline. The centrifugation and washing were repeated three times. The tubes were then allowed to dry at 4°C overnight. The antigen-antibody complex was then dissolved in 0.3 ml 0.005 N NaOH and the amount of protein in the precipitate was analysed.

Protein analysis was performed according to the Folin-ciocalteu colorimetric method described by Lowry (1951). Bovine serum albumin was used as a standard in the 50-500 µg/ml range. This method is sensitive to detect 10 µg of protein. Absorbance was read at 750 mµ in a Beckman model B spectrophotometer.

3. Conjugation of antibody with fluorescein isothiocyanate

The conjugation procedure was that of Cherry et al (1960) with modifications. The purified γ-globulin from the two rabbits was pooled and the protein concentration adjusted to 11.6 mg/ml. This solution was divided, half being kept for control purposes. An amount of Na_2CO_3 buffer (0.5M, pH 9.5) equal to 10% the volume of γ-globulin solution was added with stirring in an ice bath. This was left for two hours at 4°C, then enough fluorescein isothiocyanate (Nutritional Biochemical Corporation) was added

to give 12.5 ug FITC/mg protein. After stirring for 45 minutes at room temperature, the solution was passed through a column of Sephadex G25 (Pharmacia, Ltd.) equilibrated with 0.01M phosphate buffer, pH7. Essentially, this procedure removes the unreacted fluorescein from that conjugated with the γ-globulin (Curtain, 1961). Fifteen ml of crude conjugate were applied to the column at once. When filtering through, the fluorescent labeled antibody comes down in a discrete band which can be collected as one fraction just after the void volume. A dilution of the labeled antibody in the order of 25% was produced by this method. The unreacted fluorescein, which forms a strong band at the top of the column, was removed subsequently by washing with phosphate buffer. The labeled antibody was separated into one ml quantities and frozen at -20°C.

4. Injection of lambda carrageenan and histologic procedures

Carrageenan granulomas were produced by the procedure described by McCandless and Lehoczky-Mona (1964). The same lambda carrageenan preparation was utilized as that used for immunization and was prepared in the manner described previously. Five ml of this solution were injected subcutaneously into the ventral abdominal region of guinea pigs. The injection of this viscous solution was facilitated by the previous injection of 5 ml of air. After 5, 6, 7, 8, 12 and 14 day intervals the guinea pigs were sacrificed by ether inhalation and the granuloma, spleen, and parietal lymph nodes removed for histological examination. The tissues used in the fluorescent antibody studies were processed according to the procedure of Sainte-Marie (1962), to minimize autofluorescence. The deparaffinization procedure so described was not found to be adequate to remove all paraffin from these tissues, so the slides were

placed in two additional baths of cold xylene for 10 minutes each. Sections were cut on a rotary microtome, and were 4-6 μ thick.

Certain staining procedures were used to provide comparisons for the fluorescent antibody stained sections. Tissues were fixed either by the procedure of Sainte-Marie (1962) or in neutral buffered 10% formalin (Lillie, 1954), for 24 to 48 hours, then dehydrated in graded alcohols, cleared in xylol and embedded in paraffin. Sections were cut as for fluorescent staining. The stains used were:

a) toluidine blue, 0.25%, buffered to pH 3.2 with glycine buffer (0.25M)

b) haematoxylin and eosin

(Humason, 1962)

5. Staining procedures with FITC conjugated antibody

After processing, the tissue sections were ringed with nail polish to prevent the antibody solutions running off the slide. The fluorescent antibody was layered on the sections at various dilutions from full strength up to 1:16 antibody to phosphate buffered saline. The slides were incubated 30 minutes at 37°C in moist chambers, consisting of petri dishes lined with wet filter paper. The slides were then washed with 2 changes of phosphate buffered saline for a total of one hour. The sections were mounted in phosphate buffered glycerol. The cover slips were ringed with permount or nail polish to provide semi-permanent slides (Goldman, 1968). The following controls were used in the fluorescent antibody method:

a) The sections were incubated with unlabeled anti-lambda carrageenan antibody for 30 minutes, washed, and incubated with FITC labeled antibody for 15 minutes, washed, and mounted.

b) Unlabeled antibody was mixed with labeled antibody (1:1) and this was layered on the sections and incubated as usual.

c) Slides were incubated with unlabeled antibody alone and with phosphate buffered saline for comparison. Subsequent alterations in the staining procedure were introduced to improve the quality of staining:

a) longer incubation periods of 2 hours at 37°C.

b) more extensive washing with phosphate buffered saline.

c) incubation at 4°C overnight.

d) combination of incubation at 37°C and at 4°C.

The slides were observed in a dark room under a Leitz Orthoplan microscope which permits both phase contrast and fluorescent microscopy. For observation of fluorescence, BG3 mm and 1.3 mm excitation filters and a K530 barrier filter were used and observations made under darkfield illumination.

Absorption of the labeled antibody with tissue powder was tried. The tissue powder was prepared according to the modified method of Coons as presented in Kawamura (1969). The labeled antibody was absorbed by both dry and wet powder methods (Kawamura, 1969).

Some slides were treated with saturated bromine water to lessen autofluorescence (M. McCully, personal communication). After de - paraffinization the slides were immersed in saturated bromine water for one to 15 minutes. The slides were allowed to dry thoroughly to insure complete evaporation of the bromine. Staining was carried out by procedures already described.

6. Peritoneal cell exudate preparation

Guinea pigs were injected intraperitoneally with 5 ml 10% sterile proteose peptone solution in 0.9% NaCl, with pH adjusted to 7.4 with

NaH_2PO_4. Three days later the guinea pigs were sacrificed by ether inhalation and the peritoneum was injected with 40 ml sterile Hanks Balanced Salt Solution (Grand Island Biological) containing heparin. The abdomen was then opened under aseptic conditions and the exudate collected by sterile pipettes. The exudate was filtered through sterile gauze and then centrifuged at 1000 rpm for 10 minutes. The pelleted cells were resuspended in 90% Hank's and 10% foetal calf serum and dispensed in two ml quantities into Leighton tubes containing cover slips. These were incubated 45 minutes at 37°C to allow adhesion of the cells. The medium was then removed and the cells were washed with sterile phosphate buffered saline. To each tube was then added medium consisting of 20% foetal calf serum or 10% foetal calf serum and 10% guinea pig serum, plus 80% NCTC 109 (Grand Island Biological) and 0.8% antibiotic (Penicillin-Streptomycin Solution-5000 units penicillin and 5000 mcg streptomycin/ml - Grand Island Biological). After 24 hours the medium was changed and 10-200 µg carrageenan/ml medium added. Lambda carrageenan caused a protein precipitation which was more marked at the higher concentrations. Two different types of carrageenan were tested, lambda and an unfractionated carrageenan. Some cells were maintained in a carrageenan free medium. At different time intervals the cover slips were removed from incubation and either washed in phosphate buffered saline, air dried and stored in a dessicator at -20°C or heat killed and fixed in methanol for five minutes. The cover slips were then treated with fluorescent antibody as described previously.

In some tests, the carrageenan solution (5 ml 0.2% carrageenan in phosphate buffered saline) was injected four hours before the animal was

sacrificed. In this case the cover slips were fixed immediately after the cells had been allowed to adhere to the cover slips.

CHAPTER III

RESULTS

A. ^{14}C labeling experiments

1. Yields

The yield of crude carrageenan obtained in each of the four experiments is summarized in Table I. The higher yields in experiments #3 and #4 as compared to experiments #1 and #2 may be accounted for by the improved extraction method used in the later experiments, and greater experience with extraction procedures. Although only one batch of seaweed, taken in each of three different seasons (June, September and January), was extracted no great difference was found in the amount of carrageenan extracted.

For experiment #1 and #2, the percent dry weight of seaweed extracted for each sample is listed in Table I. Stancioff (1965) describes an extraction procedure for Chondrus crispus similar to the one used in experiment #1 and #2, in which his yield of crude carrageenan was 32.5% of the original dry weight which compares favorably with our data. Very little variation was found in the percent extracted per sample in each experiment. In experiments #3 and #4 (done according to procedure b) the standard error was ± 0.29 and ± 0.63 respectively which represents 2.5% and 5.7% of the mean extracted. In experiment #1, although only three samples were taken, the standard error was ± 0.20 which represents 3.3% of the mean extracted for that experiment.

Carrageenan extracted from Chondrus crispus

Experiment # and Sample*	Wet wt. of seaweed sample (gm)	Wt. of carrageenan extracted (mg)	% of wet wt. extracted	Dry wt. of seaweed (gm)	% of dry wt. extracted
Experiment #1					
12-0 hr	9.33	779.3	8.4	2.17	35.9
24-0 hr	10.14	789.2	7.8	2.31	34.2
48-0 hr	16.92	1324.4	7.8	3.93	30.2
Mean**			$8.0 \pm 0.29 (\pm 0.20)$		
Experiment #2					
93-3 hr	97.20	6392.1	6.6	24.5	35.5
Experiment #3					
6-2 hr	4.53	467	10.3		
10-2 hr	4.52	534	11.8		
24-2 hr	4.32	512	11.8		
34-2 hr	4.50	544	12.1		
48-2 hr	4.68	552	11.8		
48.6 hr	7.09	874	12.3		
Mean**			$11.7 \pm 0.65 (\pm 0.29)$		
Experiment #4					
6-2 hr	4.55	534	11.7		
24-2 hr	4.87	431	8.9		
34-2 hr	4.49	511	11.4		
48-0 hr	4.44	445	10.0		
48-2 hr	4.24	565	13.3		
48-6 hr	4.74	495	10.4		
Mean**			$11.0 \pm 1.4 (\pm 0.63)$		

* - Sample listed as hours of exposure to ^{14}C plus hours of chase.
** - Mean $\pm$ standard deviation($\pm$standard error of the mean).

Table I

The yields of λ- and K-carrageenan from the fractionation of the extracts are presented in Table II. As stated previously experiment #1 was fractionated according to procedure a) in Methods. In experiment #2 the 3M KCl fractionation was carried out according to method a), but method b) was used for the 0.3M fractionation of experiment #2 as well as for fractionation of experiments #3 and #4. The recovery for the fractionation is presented in Tables II and III. A statistical analysis of the data of experiments #3 and #4 is presented in Table IV. The proportions of λ- and K-carrageenan found in these experiments are quite different from those in most published data. This will be discussed subsequently.

The Smith, Cook and Neal (1954) refractionation of the KCl insoluble fractions of experiments #3 and #4 are summarized in Table V and VI. The fraction designated K_2 is a relatively pure kappa fraction. The K_3 fraction, however, probably contains a mixture of lambda and mu carrageenans. This latter fraction is an extremely small portion of the original kappa carrageenan. The average yields and statistical analysis of the refractionation are presented in Table VII.

Fractionation yield

Experiment # Sample*	Wt. of crude carrageenan mg	Molarity of KCl	λ-carrageenan KCl soluble mg	K-carrageenan KCl insoluble mg	Σ(λmg+Kmg)
Experiment #1					
12-0 hr	---				
24-0 hr	---				
48-0 hr	1311.9	3	99.4	471.4	570.8
Experiment #2					
93-3 hr	2037.2	3	235.2	1338.2	1573.4
	2992.3	0.3	138.9	2301.0	2439.9
Experiment #3					
6-2 hr	467	0.3	23.5	345.9	369.4
10-2 hr	534	0.3	23.3	419.7	443.0
24-2 hr	512	0.3	20.1	406.7	426.8
34-2 hr	544	0.3	27.6	450.3	477.9
48-2 hr	552	0.3	44.1	391.8	435.9
48-6 hr	874	0.3	93.8	534.4	628.2
Experiment #4					
6-2 hr	534	0.3	55.4	388.3	443.7
24-2 hr	431	0.3	33.5	338.4	371.9
34-2 hr	511	0.3	31.0	403.6	434.6
48-0 hr	445	0.3	23.7	330.2	353.9
48-2 hr	565	0.3	23.8	352.5	376.3
48-6 hr	495	0.3	58.9	340.0	398.9

* - Samples listed as hours of exposure to ^{14}C plus hours of chase.

Table II

Table III

Percentage yield of fractionation

Experiment # and sample*	Percent recovery	Percent λ-carrageenan of recovered	Percent K-carrageenan of recovered	Percent λ-carrageenan of total	Percent K-carrageenan of total
Experiment #1					
12-0 hr					
24-0 hr					
48-0 hr	43.5	17.4	82.6	7.53	35.8
Experiment #2					
93-3 hr	77.2**	14.9	85.1	11.3	64.5
	81.5***	5.7	94.3	4.6	77.0
Experiment #3					
6-2 hr	79.1	6.4	93.8	5.0	74.1
10-2 hr	82.9	5.3	94.8	4.4	78.7
24-2 hr	83.3	4.7	95.3	3.9	79.5
34-2 hr	87.8	5.8	94.1	5.1	82.7
48-2 hr	78.9	10.1	89.9	8.0	71.0
48-6 hr	71.8	14.9	85.0	10.7	61.0
Experiment #4					
6-2 hr	83.1	12.5	87.4	10.4	72.7
24-2 hr	86.4	9.0	90.9	7.8	78.4
34-2 hr	85.1	7.1	92.9	6.1	79.1
48-0 hr	66.5	6.3	93.8	4.2	62.3
48-2 hr	80.6	14.8	85.2	11.9	68.7
48-6 hr	79.6	6.7	93.2	5.3	74.2

* - Samples listed as hours of exposure to ^{14}C plus hours of chase.
** - Fractionated at 3M KCl, method a).
*** - Fractionated at 0.3M KCl, method b).

Average yields of fractionation by method b)

	Experiment #3			Experiment #4		
	Mean	Standard deviation	Standard error	Mean	Standard deviation	Standard error
Percent recovery	80.6	±5.3	±2.1	80.2	±7.2	±2.9
Percent λ-carrageenan	6.2	±2.6	±1.1	7.6	±3.0	±1.3
Percent K-carrageenan	74.5	±7.8	±3.2	72.5	±5.3	±2.6

Table IV

Table V

Refractionation of 0.25M KCl insoluble fraction (Smith, Cook, and Neal)

Experiment # and sample*	Amount of 0.25M KCl insoluble K_1 mg	Amount insoluble at 0.15M KCl K_2** mg	Amount soluble at 0.15MKCl K_3*** mg	$\sum_{mg}(K_2+K_3)$
Experiment #3				
6-2 hr	85.3	87.5	18.3	105.8
10-2 hr	87.4	96.3	7.5	103.8
24-2 hr	84.2	85.6	5.4	91.0
34-2 hr	86.0	84.5	8.5	93.0
48-2 hr	84.7	98.3	8.2	106.5
48-6 hr	88.8	92.2	8.0	100.9
Experiment #4				
6-2 hr	85.2	101.9	6.6	108.5
24-2 hr	85.3	116.3	6.4	122.7
34-2 hr	85.8	95.9	3.0	98.9
48-0 hr	84.4	83.5	4.7	88.2
48-2 hr	84.7	104.8	5.7	110.5
48-6 hr	84.5	88.9	8.5	97.1

* - Samples listed as hours of exposure to ^{14}C plus hours of chase.
** - K_2 represents a purer kappa carrageenan fraction than K_1.
*** -K_3 represents a fraction consisting of a mixture of λ- and μ-carrageenan.

Table VI

Percentage yields of refractionation (Smith, Cook, and Neal)

Experiment # and sample*	Percent recovery	Percent K_2 of recovered	Percent K_3 of recovered	Percent K_2** of unfractionated	Percent K_3** of unfractionated
Experiment #3					
6-2 hr	125	82.7	17.3	76.5	16.0
10-2 hr	119	92.8	7.2	82.1	6.4
24-2 hr	108	94.1	5.9	75.8	4.8
34-2 hr	108	90.9	9.1	73.2	7.4
48-2 hr	126	92.3	7.7	86.5	7.2
48-6 hr	103	92.1	7.9	78.0	6.7
Experiment #4					
6-2 hr	127	93.9	6.1	86.7	5.6
24-2 hr	144	94.8	5.2	98.8	5.4
34-2 hr	114	97.0	3.0	81.1	2.5
48-0 hr	104	94.7	5.3	71.7	4.3
48-2 hr	132	94.8	5.2	89.7	4.9
48-6 hr	115	91.2	8.8	76.1	7.3

* - Sample listed as hours of exposure to ^{14}C plus hours of chase.

** - From Table IV K_1 represents in experiment #3, 74.5±7.8% of unfractionated carrageenan (for experiment #4, 72.5±5.3%). The unfractionated weights are calculated from this and from the amount of K_1 used for the fractionation.

Average yield from Smith, Cook, and Neal refractionation

	Experiment #3			Experiment #4		
	Mean	Standard deviation	Standard error	Mean	Standard deviation	Standard error
Percent K_2 of total	78.6	$\pm$5.2	$\pm$2.1	84.0	$\pm$9.8	$\pm$4.0
Percent K_3 of total	8.1	$\pm$4.0	$\pm$1.6	5.0	$\pm$1.6	$\pm$0.65

Table VII

2. Specific Activity

Specific activity is an expression denoting the concentration of labeled atoms in the material under study, in this case ^{14}C dpm/µg galactose (Table VIII). To obtain specific activity in terms of µg carrageenan rather than galactose, one must take into account that lambda carrageenan contains about 35% sulphate, kappa carrageenan about 30%. It should also be borne in mind that the absorbance of 3,6-anhydrogalactose is only 0.92 that of galactose, which would necessitate a significant correction for kappa carrageenan which has about 29% 3,6-anhydrogalactose. Since sulphate and anhydrogalactose vary from sample to sample, however, the data are expressed more simply in terms of galactose units. In order to compare the specific activities of the different experiments, the values were corrected to a similar isotope concentration, viz. 0.1 mc/100 ml seawater, i.e., the results from experiment #1 were multiplied by 5 and those from experiment #2 by 2.5. Even with this manipulation, the fractions are not strictly comparable since extraction and fractionation methods were different and therefore the fractions of experiment #1, #2 and #3, #4 are not of equal purity.

In experiment #2, two different fractionations were carried out on the same carrageenan. The first at 3M KCl by method a) gave a lambda fraction with a specific activity of 15.7 dpm/µg galactose; the kappa fraction was refractionated at 0.25M KCl and gave an insoluble fraction with a specific activity of 2.03 dpm/µg galactose and a soluble fraction with 7.95 dpm/µg galactose. The soluble fraction represents about 3% of the original kappa fraction in this case. The fractionation at 0.3M KCl by method b) gives a much lower percentage of lambda carrageenan with a much higher specific activity; it is obviously a purer fraction.

Table VIII

Specific activity of the carrageenan fractions (corrected)

Experiment # and sample*	Specific activity dpm/µg gal				
	Crude	λ	K_1	K_2	K_3
Experiment #1**					
12-0 hr	2.45				
24-0 hr	3.04				
48-0 hr	9.50	17.90	3.40		
Experiment #2***					
93-3 hr (3M)	12.90	15.70			
(0.3M)		46.90	1.52		
Experiment #3					
6-2 hr		5.72	0.085	0.170	1.05
10-2 hr		10.10	0.226	0.180	1.24
24-2 hr		7.80	0.252	0.210	1.23
34-2 hr		12.75	0.620	0.635	2.01
48-2 hr		13.05	0.575	0.538	2.01
48-6 hr		12.40	0.418	0.484	1.57
Experiment #4					
6-2 hr		2.70	0.252	0.185	0.695
24-2 hr		11.10	0.278	0.560	1.810
34-2 hr		16.50	0.995	0.462	2.170
48-0 hr		17.10	1.180	0.687	2.280
48-2 hr		14.80	0.788	0.678	2.050
48-6 hr		19.40	0.862	0.925	2.280

* - Samples listed as hours of exposure to ^{14}C plus hours of chase
** - Actual value X5
*** - Actual value X2.5

The specific activities for experiment #3 and #4 are plotted against time in Figures 1-4. In Figure 1 a rapid incorporation of ^{14}C into λ-carrageenan occurred in the first ten hours, then the rate decreased during the next twelve hours, increased in the next twelve, and leveled off in the last twelve. Both periods of leveling off occurred during the dark period. This pattern is also seen in kappa carrageenan (K_1). Note that the scale used in plotting kappa carrageenan in Figure 1 and 2 is expanded by an order of magnitude. A similar graph of data in experiment #4 is presented in Figure 2. There the pattern is less obvious since the sample at hour 10 was lost; but one can postulate that the increase would be rapid up to 10 hours then level off during hour 12 to 24 (based on additional data from other labeling experiments in McCandless and Richer (1971), see discussion).

There was no great difference in specific activity curves of experiment#3 and #4, carried out with a light/dark cycle and continuous light, respectively. At later time periods, the continuous light might eventually cause bleaching of the photosynthetic pigments and thus affect the rate of incorporation. The seaweed collected in winter has a lower specific activity than that collected in summer or fall (McCandless and Richer, 1971) but this will be discussed later. In all experiments the incorporation of ^{14}C into kappa carrageenan was much slower than into lambda carrageenan. In Figures 3 and 4 are plotted the results of the refractionation of the kappa carrageenan by the method of Smith, Cook and Neal (1954), using a KCl concentration of 0.15M rather than 0.3M to isolate kappa carrageenan. For kappa carrageenan, the diurnal rhythm seen in lambda carrageenan is not found in all experiments. The variation in specific activity is there but does not seem to follow a pattern. The K_2

fraction rhythm (purer kappa fraction) shows the diurnal rhythm in experiment #3 but not in experiment #4. It was also not apparent in the K_2 fractions of other experiments reported by McCandless and Richer (1971). In these experiments, the specific activity of kappa carrageenan was higher in seaweed collected in autumn than at any other time.

The fraction designated K_3 is not considered to be a pure fraction but a mixture of some lambda, and mu if present; therefore, little meaning can be attributed to the specific activity data of this fraction.

The rate of biosynthesis of the polysaccharide carrageenan is not known; i.e., the time lapse between the assimilation of ^{14}C by the algae and its appearance in the polysaccharide carrageenan. Also the rate of breakdown of carrageenan is not known. In order to obtain carrageenan of a high specific activity, both these processes are important and they can be assessed by "chasing" the seaweed samples in non-radioactive seawater. In experiments #3 and #4 different lengths of chase were given to the 48 hour samples. As can be seen in Table VIII, the data are sufficiently variable that no pattern can be detected in samples given either no chase, a two hour chase, or a six hour chase; however, it can be said that at 48 hours of incorporation the effect of chase up to six hours on specific activity is not very significant.

3. Chemical analysis

Carrageenan is known to be hygroscopic. If carrageenan is dried for 24 hours at 56°C in vacuo before weighing, there is about a 20% weight loss which is rapidly regained if the sample is left exposed to air. Measurement of content of galactose represents only about 70% of the weight of the polymer (30% of the weight is due to sulphate). Therefore, the galactose content of a non-dried sample should be about 50% of the weight of carrageenan. Calculated values of 30 to 60% were in fact found for the samples (λ, K_1, K_2, K_3) of experiments #3 and #4. Presumably, the variation is due to differences in water absorption by the different samples as well as to the variation in sulphate content. This variation supports the use of dpm/μg galactose as a measurement of specific activity, as already mentioned.

The resorcinol test for 3,6-anhydrogalactose was carried out on material from experiment #1; 48 hour kappa and lambda carrageenan were found to contain 27% and 1.3% 3,6-anhydrogalactose respectively. A sample of 3M KCl insoluble kappa carrageenan from experiment #2 was refractionated at 0.25M KCl; the fraction insoluble at 0.25M KCl had 27.4% 3,6-anhydrogalactose, i.e., it contained more kappa carrageenan than the soluble portion (16.5% 3,6-anhydrogalactose).

To test for possible hydrolysis of λ-carrageenan during extraction and fractionation, a sample of the 3M lambda carrageenan was tested for reducing sugar. No reducing sugar was present in this sample indicating hydrolysis had not occurred. No contamination with protein was found in 0.3M and 3M kappa and lambda carrageenan from experiment #2 when they were tested by the Lowry procedure (1951).

To identify the polysaccharide units into which radioactivity had been incorporated, the hydrolysate of the 0.3M KCl and 3M KCl lambda carra-

geenan fractions from experiment #2 were chromatographed. Sample chromatograms and radioactivity measurements are shown in Figures 5, 6 and 7. The hydrolysate of each sample gave three positive regions when tested for reducing sugar. One had an Rf comparable to galactose, one had an Rf comparable to either xylose or fucose on most chromatograms. The best separation of xylose and fucose was obtained in pyridine, ethanol, water. In Figure 7 the Rf of spots 2 and 7 was comparable to xylose. Chromatograms run in butanol, ethanol, water - 3% cetyl pyridinium bromide also gave an Rf similar to xylose. A third positive region near the solvent front was also found.

The recovery of radioactivity obtained by counting the strips amounted to only 20-30% of the total applied. The highest count was found at the origin, which would indicate incomplete hydrolysis. This spot was faintly positive to toluidine blue in the case of the 0.3M lambda carrageenan hydrolysate. The spots near the origin in Figure 6 also suggest that hydrolysis was incomplete and left various size polysaccharides. The highest radioactivity was found in the part identifiable as xylose; due to the incomplete hydrolysis most of the galactose would be found at or near the origin, and in bound form would not give a positive test for reducing sugar.

The hydrolysate was chromatographed against a hydrolysate of lambda carrageenan, provided by Marine Colloids, Inc. This is shown in Figure 8; both hydrolysates are composed of various size polysaccharides and oligosaccharides as well as the monosaccharides.

Dialysis of the radioactive carrageenans prepared in these experiments did not result in any significant loss of activity. The label was present in large enough units that it did not pass through the dialyzing membrane.

B. Metabolism experiments

The breakdown of ^{14}C lambda carrageenan was studied in three guinea pigs, after its subcutaneous injection. The first animal was injected with 2.5 ml of a 1% (by weight) solution of 48 hour lambda carrageenan prepared in experiment #1. From the specific activity, the total radioactivity injected was about 27,000 cpm. Samples of urine, feces, and CO_2 were taken at 24, 48, 72 and 96 hours. No activity was found in any of the samples taken at these times. After 14 days the guinea pig was sacrificed and parietal lymph node, spleen and granuloma tissue were taken for histological processing. The granulation tissue was minimal, but sections stained with toluidine blue revealed the presence of metachromatic material in the macrophages. The tissue was typical of that produced on injection of lambda carrageenan as described by Williams (1957).

The second guinea pig was injected with 2 ml of a 1% (by weight) solution of 3M lambda carrageenan prepared in labeling experiment #2. A total of 18,876 cpm was injected. The first samples were taken 22 hours after injection; no activity was found in the expired CO_2, a 0.05 ml sample of urine contained 9.3 cpm. The total volume of urine collected at this time was 19 ml, hence this would represent 18.7% of the total counts injected. Samples were taken again at 46 and 69 hours but no further activity was detected. This guinea pig was sacrificed seven days after injection. The same tissues as in the first were taken for histological and autoradiographic examination. The granuloma had a wet weight of 0.4695 g. Metachromatic material was seen in the macrophages of the granulation tissue.

The third guinea pig was injected with 31.5 mg of 0.3M lambda

carrageenan prepared from the ^{14}C carrageenan of labeling experiment #2. This preparation has a specific activity of 15.9 dpm/μg, a total of 65,190 cpm was injected. The first sample was taken after 24 hours. No counts were found in the NaOH. A total of 60 ml of urine was collected at this time, a 0.05 ml sample had 8.6 cpm, this represents 15.9% of the total injected. Later samples taken at 48 and 72 hours showed no activity. The guinea pig was sacrificed after seven days. The granuloma found was large and spread over a large part of the abdominal region. The wet weight of the granulation tissue was about 9 grams.

C. Autoradiography

Autoradiography was performed on the tissues from the three guinea pigs used in the metabolism experiments. An additional guinea pig was injected subcutaneously with a lambda carrageenan supplied by Dr. E. L. McCandless, prepared by the method of Stancioff (1965). This carrageenan had a specific activity of 27.6 dpm/μg galactose. Twenty-six mg were dissolved in 3.5 ml of phosphate buffered saline, 3 ml of this solution were injected; therefore, the animal received a total of 2.4×10^5 cpm. The animal was sacrificed 14 days after injection.

None of the autoradiograms prepared gave positive results even though granulomas did result from the injections and metachromasia could be seen in the tissue macrophages.

D. Fluorescent Antibody

1. Response to immunization

Precipitation reactions were carried out using γ-globulin precipitated from rabbit serum with ammonium sulphate and redissolved in phosphate buffered saline to the original volume of the serum. Precipitation reactions were carried out both on the pre-injection γ-globulin and on the γ-globulin from serum taken 4 days after the final injection in the immunization procedure. Quantitative precipitation curves are presented in Figure 9. A maximum of 2.06 mg antibody/ml was precipitated from the post-immunization serum of rabbit #1 as compared to 0.81 mg/ml from the pre-injection serum. For rabbit #2, the maximum antibody precipitated from the post-immunization serum was 2.90 mg/ml. The control for rabbit #2 was 0.78 mg/ml.

2. Histological observations

Seven granulomas, resulting from the injection of λ-carrageenan, were excised 5, 6, 7, 8, 12, and 14 days post injection. Sections stained with haematoxylin and eosin (H and E) and with toluidine blue were studied and provided a general description of the granuloma and a basis for comparison with sections treated with fluorescein (FITC) labeled antibody.

The gross size of granuloma remained relatively constant regardless of the ages at which they were excised; however, the tissue at the earliest time was soft and poorly outlined and as fibrosis proceeded the tissue became firmer and easily excisable.

The histology of the granulomas presented a picture of gradual fibrosis proceeding from the periphery towards the central area of the tissue. At the earliest time (5 days), cellular infiltration of the injected material had occurred and by 7 days a distinct cellular peripheral area was present. In the granulomas aged 12 and 14 days, fibrosis had proceeded so that the central area was almost completely occluded.

The 7 day granuloma was taken as representative of the young granuloma tissue. In sections of granulomas aged 7 and 8 days, and stained with H and E, the central region appeared empty except for a few cells. The tissue became progressively more cellular in the sections taken towards the periphery of the granuloma. Increasing numbers of collagen fibers were seen in the peripheral region. These were randomly dispersed between the cells in tissue closer to the center; in contrast in the more mature areas towards the periphery, the cells and fibers were arranged in characteristic whorl patterns. The fibers occupied about 50% of the area of this mature tissue. Toluidine blue staining showed the presence of a metachromatic substance located extracellularly in the peripheral region (Figure 13).

It should be noted that in the pictures of the toluidine blue stained sections, a pronounced yellow color was produced by the photographic setup and in printing from the Kodachrome II slides. As a result, the orthochromatic stained tissue elements appear gray instead of blue and the metachromasia reddish rather than the purple observed on the slides of tissue. However, the metachromasia is still readily distinguishable on the prints. With H and E staining, the central area appeared as a large empty space but toluidine blue stained this area strongly metachromatic.

The major cell types in the granulomas, ages 5 to 8 days, were fibroblasts, and macrophages. Fibroblasts and macrophages could not be differentiated after H and E staining. Polymorphonuclear leukocytes were also seen, but their number had already decreased in the 7 and 8 day granulomas as compared to the 5 day. However, staining with toluidine blue showed cells containing metachromatic material, considered to be macrophages, and cells without metachromatic material most of which were interpreted as being fibroblasts. Intracellular metachromatic material can be seen in Figures 13, 14, 16, and 19. A higher proportion of the cells found toward the center contained metachromatic material than in the more peripheral areas.

The granulomas aged 12 and 14 days showed an increase in amount of collagen and in numbers of cells. Polymorphonuclear leukocytes had decreased dramatically in numbers concomitant with an increase in the other cell components. Toluidine blue staining indicated an overall increase in numbers of macrophages and much decreased amounts of extracellular metachromasia.

The fluorescent antibody staining of the granuloma showed the fluorescence localized in the cytoplasm of cells (Figure 10). Figure 11

shows that reaction seen in Figure 10 was blocked by exposing the section to unlabeled antibody prior to exposing it to labeled antibody, while Figure 12 shows a section exposed only to unlabeled antibody. The intensity of some of the fluorescence is greater in the stained than in the two sections used as controls. The very bright fluorescence in Figure 10 seems to be associated with the cells. In many cases, the cell boundaries were difficult to define and therefore, localization of the fluorescence within the cells was not entirely conclusive, but in all cases the fluorescence was attached to or found within the cells. A more intense fluorescence was sometimes visible in the area immediately surrounding the nucleus (Figures 16 and 18). It was also difficult to differentiate between fibroblasts and macrophages after fluorescent antibody staining; therefore, the identity of the cells exhibiting fluorescence can not be limited to either of the two kinds of cells. Toluidine blue stained sections of the same tissue are shown for comparison in Figures 13 and 14. Correlation between the metachromasia in the toluidine blue stained sections and the localization of the fluorescence was not as direct as expected. However, by comparing the fluorescent antibody stained sections with toluidine blue stained sections, it can be concluded that the intense metachromasia associated with the cells is due to the presence of carrageenan within their cytoplasm.

The fluorescent staining pattern of the 12 and 14 day granulomas did not appear to differ from that of the younger tissue. The main difference of the older as compared to the younger granuloma, when stained with toluidine blue, was a lessening of the extracellular metachromasia; no significant extracellular fluorescence was seen even at the earlier times.

The staining of the spleen and parietal lymph nodes of carrageenan injected guinea pigs did not show any specific fluorescence discernable from its controls.

Due to the difficulties of interpreting the fluorescent staining of such a complex tissue as the granuloma, smears of peritoneal cells were examined with fluorescent antibody. Such a system would be useful in testing the specificity of the antibody preparation for the specific fraction against which it was produced, since K-carrageenan does not cause formation of a granuloma. Judging from intracellular metachromasia, carrageenan is presumably taken up by cultured peritoneal cell exudates (Allison et al, 1966) and by peritoneal cells in vivo. Carrageenan was injected into the abdomen at appropriate times before cell harvest. In this laboratory, the cells incubated with λ-carrageenan did show a greater degree of fluorescence than the control cells (incubated without carrageenan). However, the control slides treated with fluorescent anti-lambda carrageenan antibody showed a greater fluorescence than unstained control cells which would indicate a degree of non-specificity in the antibody preparation. The results with the peritoneal cells are questionable as there was evidence of deterioration of the fluorescent antibody preparation by this time. These studies were terminated because of the failure of rabbits subsequently injected in this laboratory to produce specific antibody (see discussion).

There were technical problems associated with fluorescent antibody techniques as applied to the carrageenan granuloma. Different methods for improving the staining were tried. When sections of carrageenan granuloma were observed using fluorescence microscopy, the tissues showed a greenish autofluorescence which interfered with the interpretation of results. The fixation procedure of Sainte-Marie (1961) did not

eliminate autofluorescence in any of the sections observed, although autofluorescence was reduced as compared with the tissues fixed in neutral buffered formalin. The alcohol fixation of Sainte-Marie (1961) does prevent the loss of carrageenan from the tissues. Carrageenan is water soluble and some is lost during an aqueous fixation procedure. The toluidine blue stained sections of tissue fixed in alcohol and in formalin confirm this by the presence of more intense metachromasia in the alcohol fixed tissues.

Bromine treatment, which was suggested as a method for reducing autofluorescence, did not do so to any appreciable extent and caused tissue damage at longer exposures; therefore its use was discontinued. Absorption of the fluorescent antibody with tissue powder did not improve the quality of staining but resulted in an overall loss in intensity of staining. Dilution of the fluorescent antibody did in some cases improve the staining by lessening what could be interpreted as a non-specific staining of the tissue components. This can be seen by comparing Figure 16 with Figure 15 and to a lesser extent Figure 18 with Figure 15. No improvement in staining was noted by altering the incubation time or temperature. The long washing time after staining was necessary to clear the slides of "unreacted" FITC antibody. Of the various control techniques used, incubating the sections with unlabeled antibody and then with the labeled antibody was found to be more effective than incubation with mixed labeled and unlabeled preparations.

Figure 1

Specific activity time curve of lambda and kappa (K_1) carrageenan from experiment #3.

Light conditions: 12hr light/12hr dark

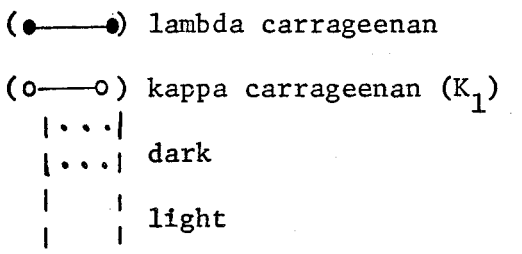


Figure 1

λ -carrageenan DPM/μg. galactose
(●——●)
K carrageenan DPM/μg. galactose
(o——o)
Time (hours)
2
4
6
8
10
12
14
16
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
5
10
20
30
40
50

Figure 2

Specific activity time curve of lambda and kappa (K_1) carrageenan from experiment #4.

Light conditions: continuous light.

(●——●) lambda carrageenan

(○——○) kappa carrageenan (K_1)

Figure 2

λ-carrageenan DPM/μg. galactose
(● ——— ●)
13
16
14
12
10
3
6
4
2
K-carrageenan DPM/μg. galactose
(o ——— o)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
5
10
20
30
40
50
Time (hours)

Figure 3

Experiment #3. Specific activity time curve of kappa carrageenan (K_1) refractionation products.

Smith, Cook, and Neal refractionation.

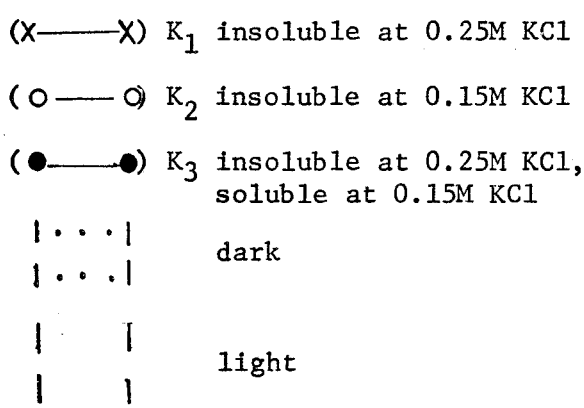


Figure 3

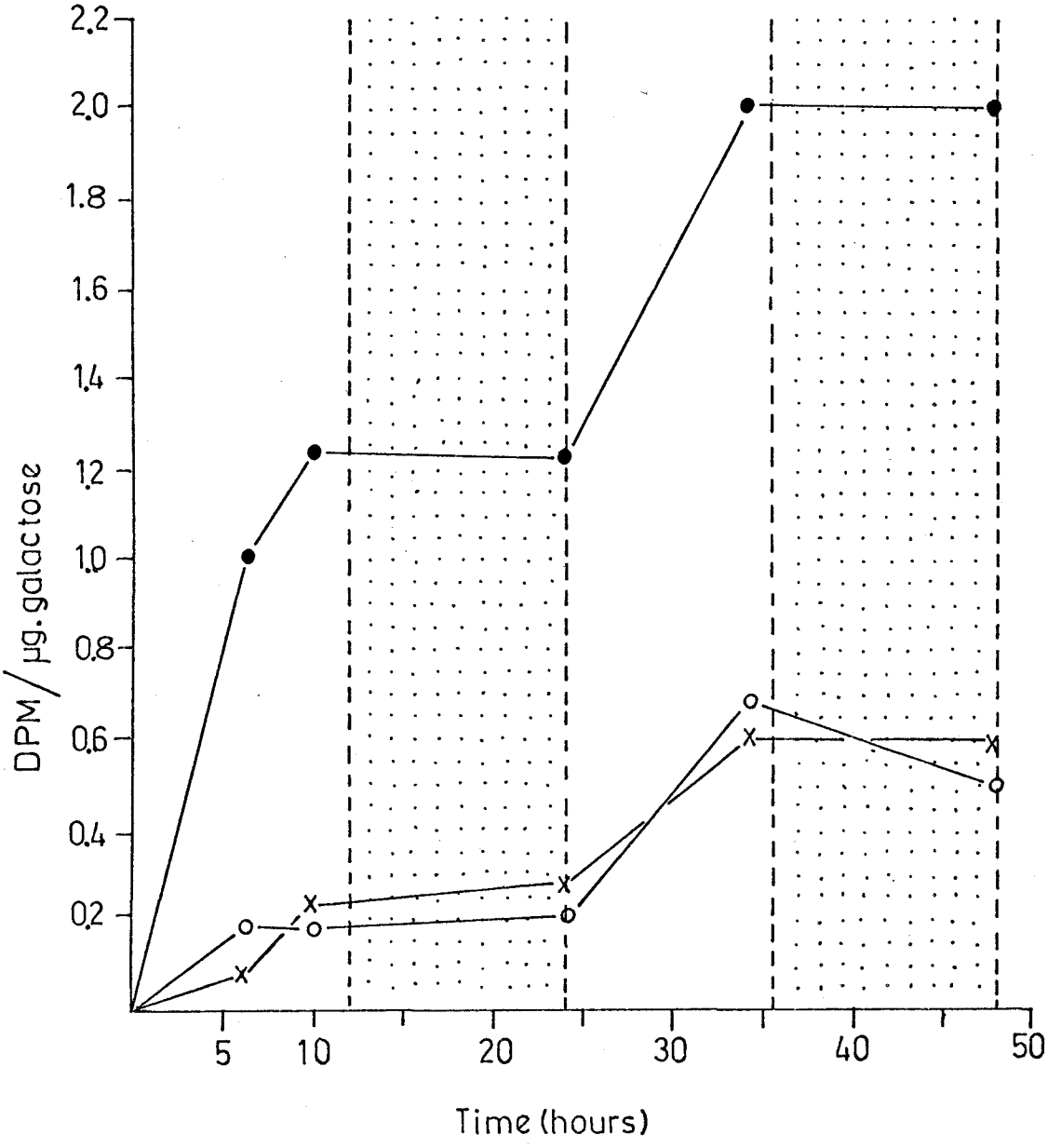

2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
DPM / µg. galactose
5
10
20
30
40
50
Time (hours)

Figure 4

Experiment #4. Specific activity time curve of kappa carrageenan (K_1) refractionation products.

Smith, Cook, and Neal refractionation. Continuous light.

(x——x) K_1 insoluble at 0.25M KCl

(o——o) K_2 insoluble at 0.15M KCl

(●——◔) K_3 insoluble at 0.25M KCl
soluble at 0.15M KCl

Figure 4

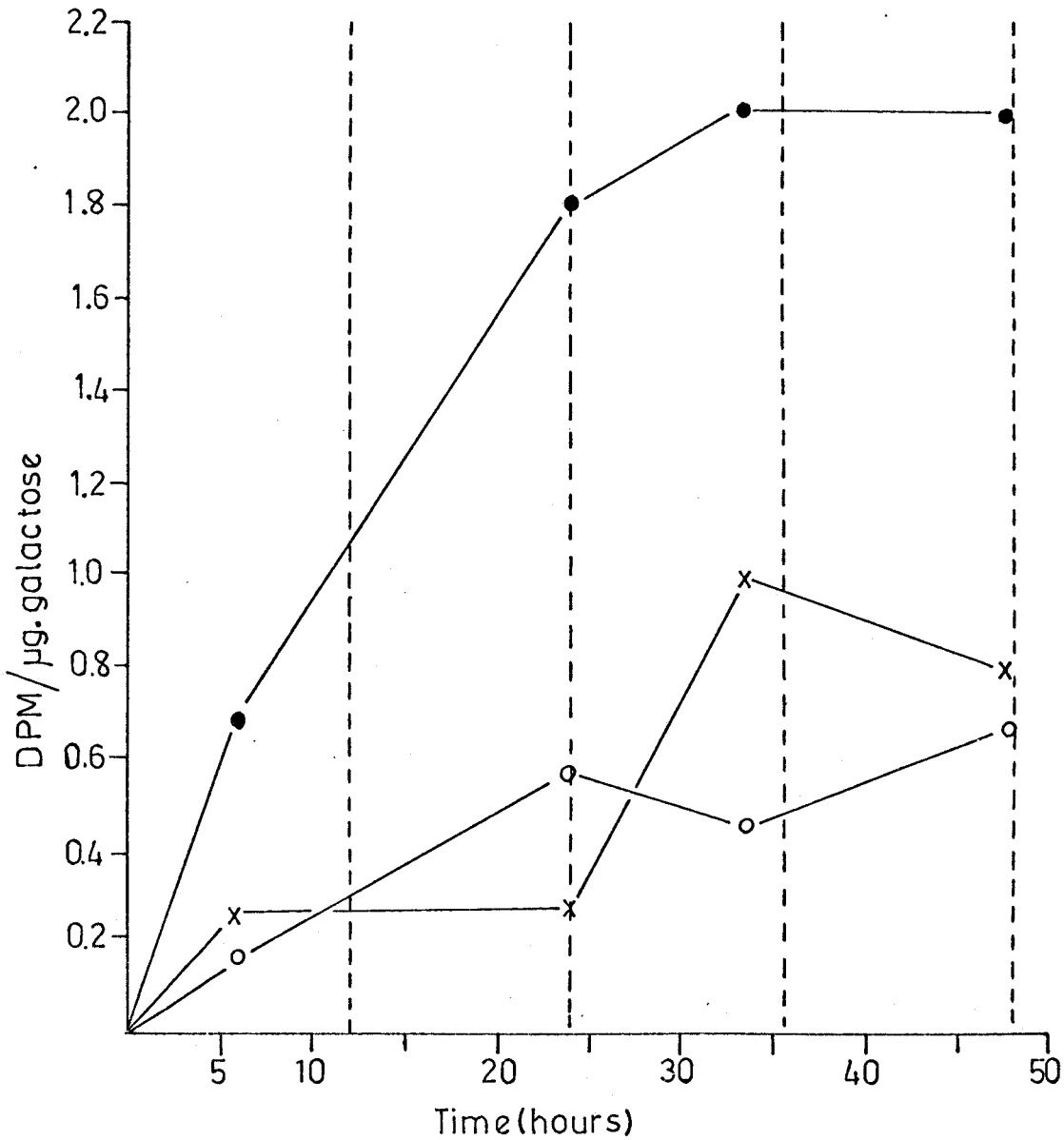

2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
DPM/µg. galactose
5
10
20
30
40
50
Time (hours)

Figure 5

Chromatogram of 0.3M and 3.0M KCl soluble (lambda) carrageenan hydrolysate. Carrageenan from experiment #2. The solvent was butanol:ethanol:H_2O, 3:1:1. Development in 3% anisidine hydrochloride in butanol. Numbers indicate spots taken for counting.

S.F. - solvent front

Fuc. - fucose

Xyl. - xylose

Gal. - galactose

Glu. - glucose

Radioactivity data (corrected for background)

Sample	Radioactivity
3.0M KCl soluble 7	14.2cpm
1	8.4cpm
2	15.9cpm
8	7.0cpm
3	2.6cpm
0.3M KCl soluble 9	50.6cpm
4	27.0cpm
5	25.6cpm
10	17.3cpm
6	4.4cpm
11	background

Total activity applied:

for 0.3M KCl λ -0.05ml- 488.8cpm
for 3.0M KCl λ -0.05ml- 190.7cpm

Total activity recovered

for 0.3M KCl λ -, 97.9cpm (20%)
for 3.0M KCl λ -, 48.1cpm (25%)

Figure 5

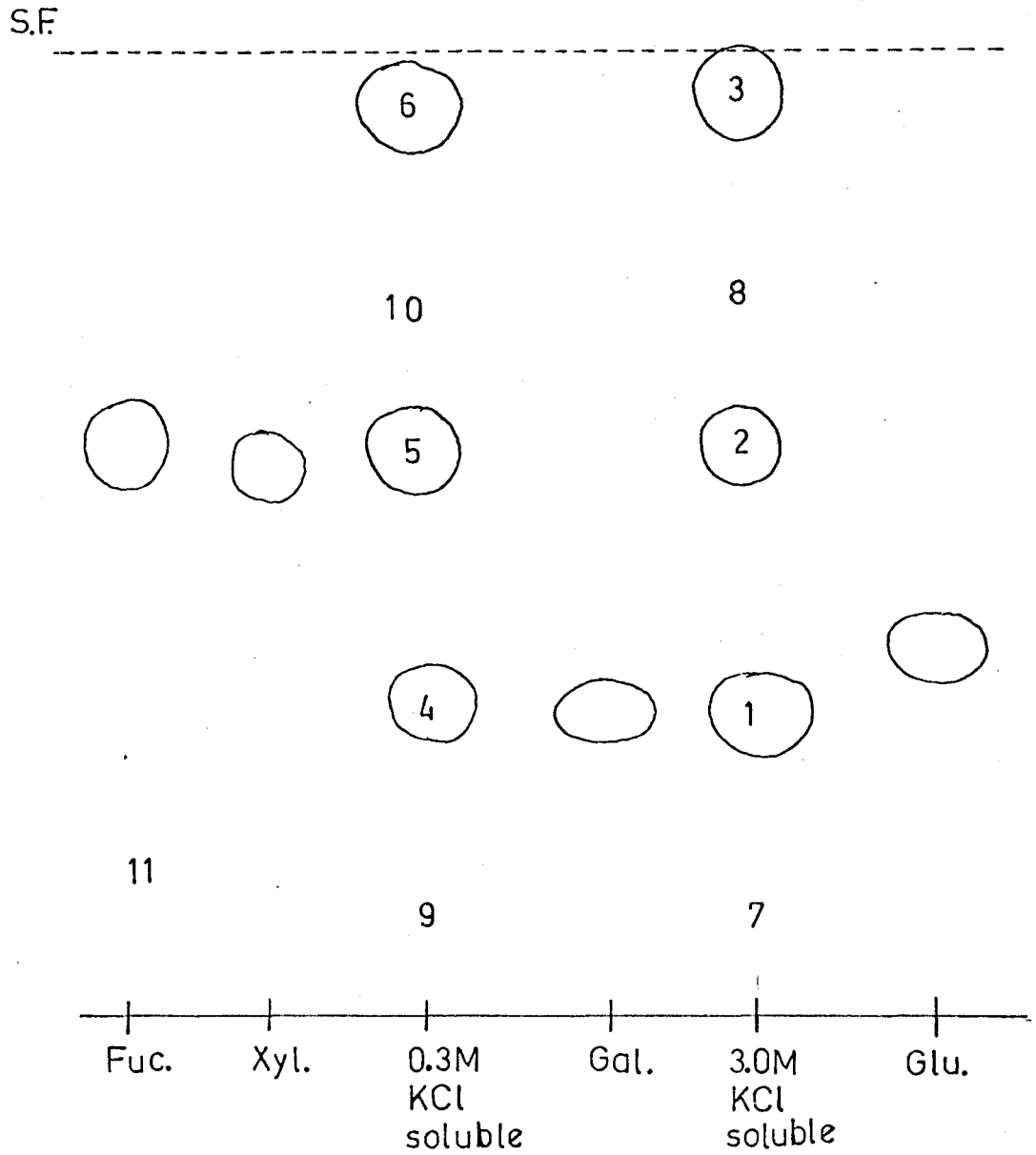

S.F.
6
3
10
8
5
2
4
1
11
9
7
Fuc.
Xyl.
0.3M KCl soluble
Gal.
3.0M KCl soluble
Glu.

Figure 6

Chromatogram of 0.3M and 3.0M KCl soluble (lambda) carrageenan hydrolysate. Carrageenan from experiment #2. The solvent was pyridine:ethanol:H_2O, 6:4:3. Development in 3% anisidine hydrochloride in butanol. Numbers indicate spots taken for counting.

S.F. - solvent front

Fuc. - fucose

Xyl. - xylose

Gal. - galactose

Glu. - glucose

Radioactivity data (corrected for background)

Sample		Radioactivity
3.0M KCl soluble	1	8.3cpm
	2	17.3cpm
	3	1.9cpm
	4	2.5cpm
	5	18.9cpm
0.3M KCl soluble	6	14.6
	7	28.2
	8	4.7
	9	0
	10	105.5
	11	background

Total activity applied:

for 0.3M KClλ - 0.05ml- 488.8cpm
for 3.0M KClλ - 0.05ml- 190.7cpm

Total activity recovered:

for 0.3M KCl - λ, 153.0cpm (31.4%)
for 3.0M KCl - λ, 48.9cpm (25.6%)

Figure 6

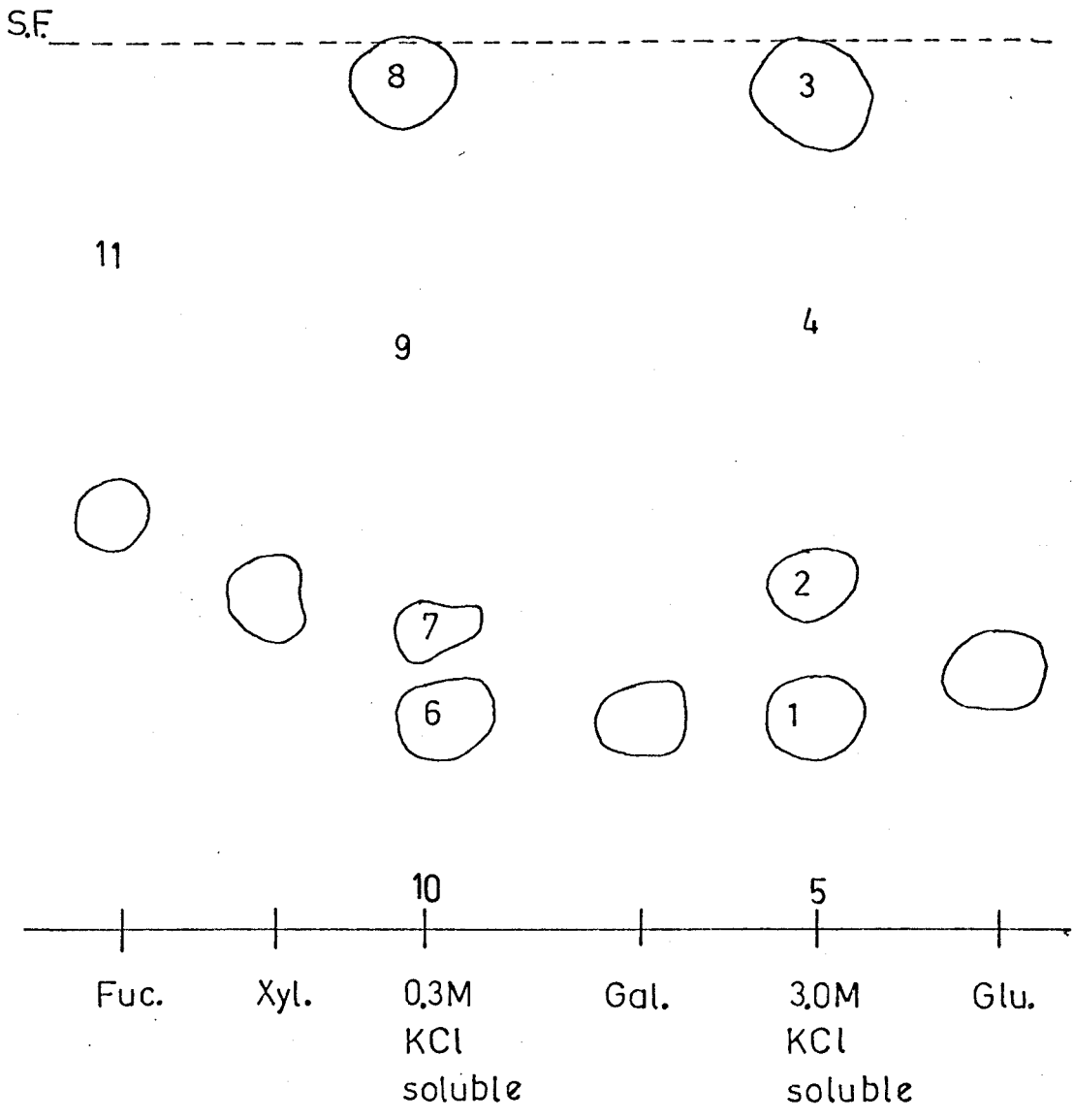

S.F.
8
3
11
9
4
2
7
6
1
10
5
Fuc.
Xyl.
0.3M KCl soluble
Gal.
3.0M KCl soluble
Glu.

Figure 7

Chromatogram of 0.3M and 3.0M KCl soluble (lambda) carrageenan hydrolysate. Carrageenan from experiment #2. The solvent was pyridine:ethanol:H_2O, 6:4:3. Development in 3% anisidine hydrochloride in butanol. Numbers indicate portions taken for counting.

S.F. - solvent front

Gal. - galactose

Glu. - glucose

Xyl. - xylose

Fuc. - Fucose

Radioactivity data (corrected for background)

Sample	Radioactivity
1	58.5cpm
2	31.7cpm
3	7.7cpm
4	7.9cpm
5	9.8cpm
6	5.2cpm
7	19.1cpm
8	4.0cpm
9	0.6cpm
10	taken as background

Total activity applied, 0.05ml-488.8cpm
Total activity recovered, 144.5cpm (29.5%)

Figure 7

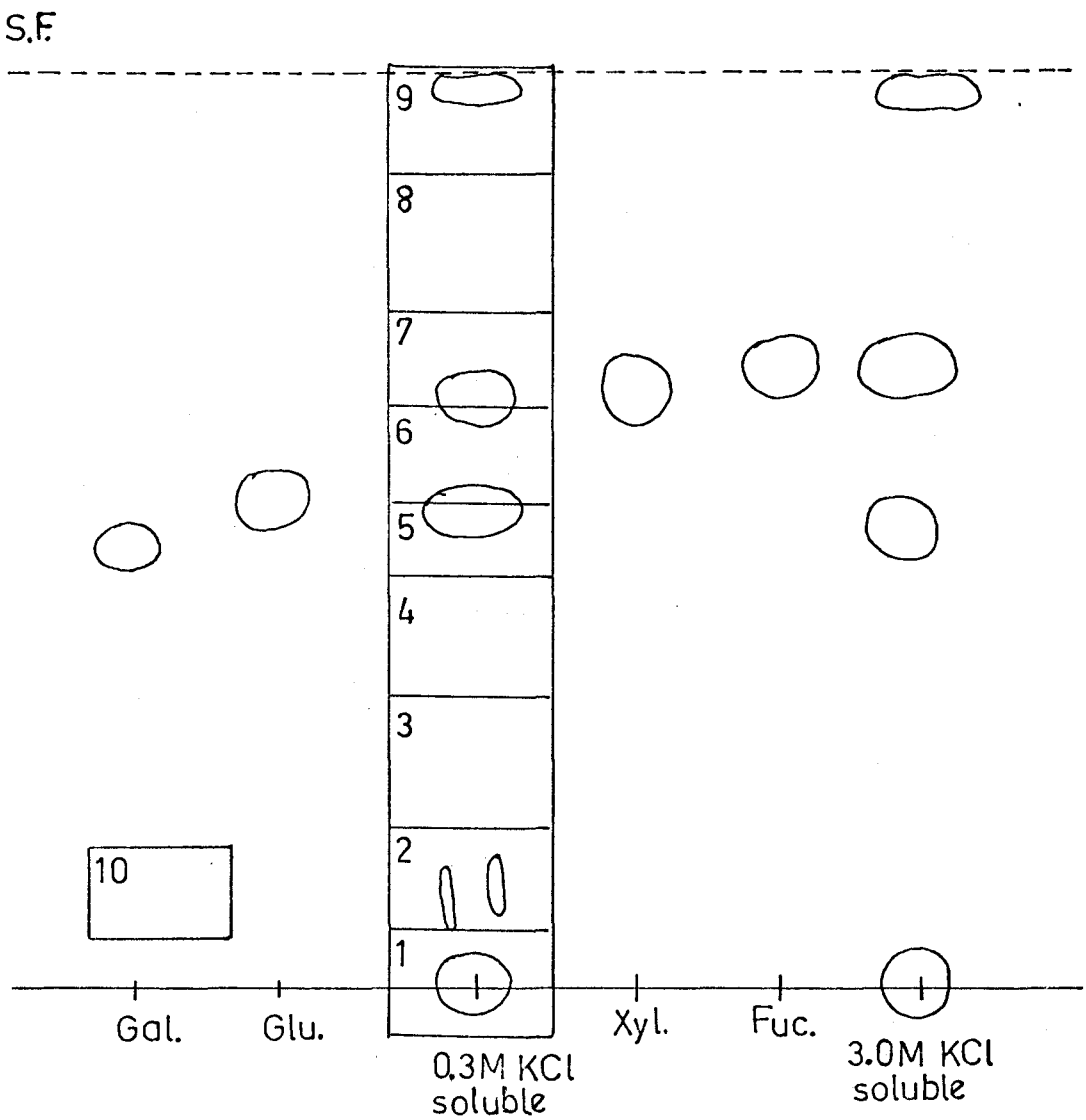

S.F
9
8
7
6
5
4
3
2
1
10
Gal.
Glu.
0.3M KCl soluble
Xyl.
Fuc.
3.0M KCl soluble

Figure 8

Chromatogram of 0.3M KCl soluble (lambda) carrageenan (experiment #2) and lambda carrageenan (Marine Colloids, Inc.)

The solvent was pyridine:ethanol:H_2O, 6:4:3.

Developed using silver nitrate.

S.F. - solvent front

M.C. - Marine Colloids, Inc. lambda carrageenan hydrolysate

0.3M KCl soluble - 0.3M KCl soluble lambda carrageenan from experiment #2. hydrolysate

Gal. - galactose

Xyl. - xylose

Figure 8

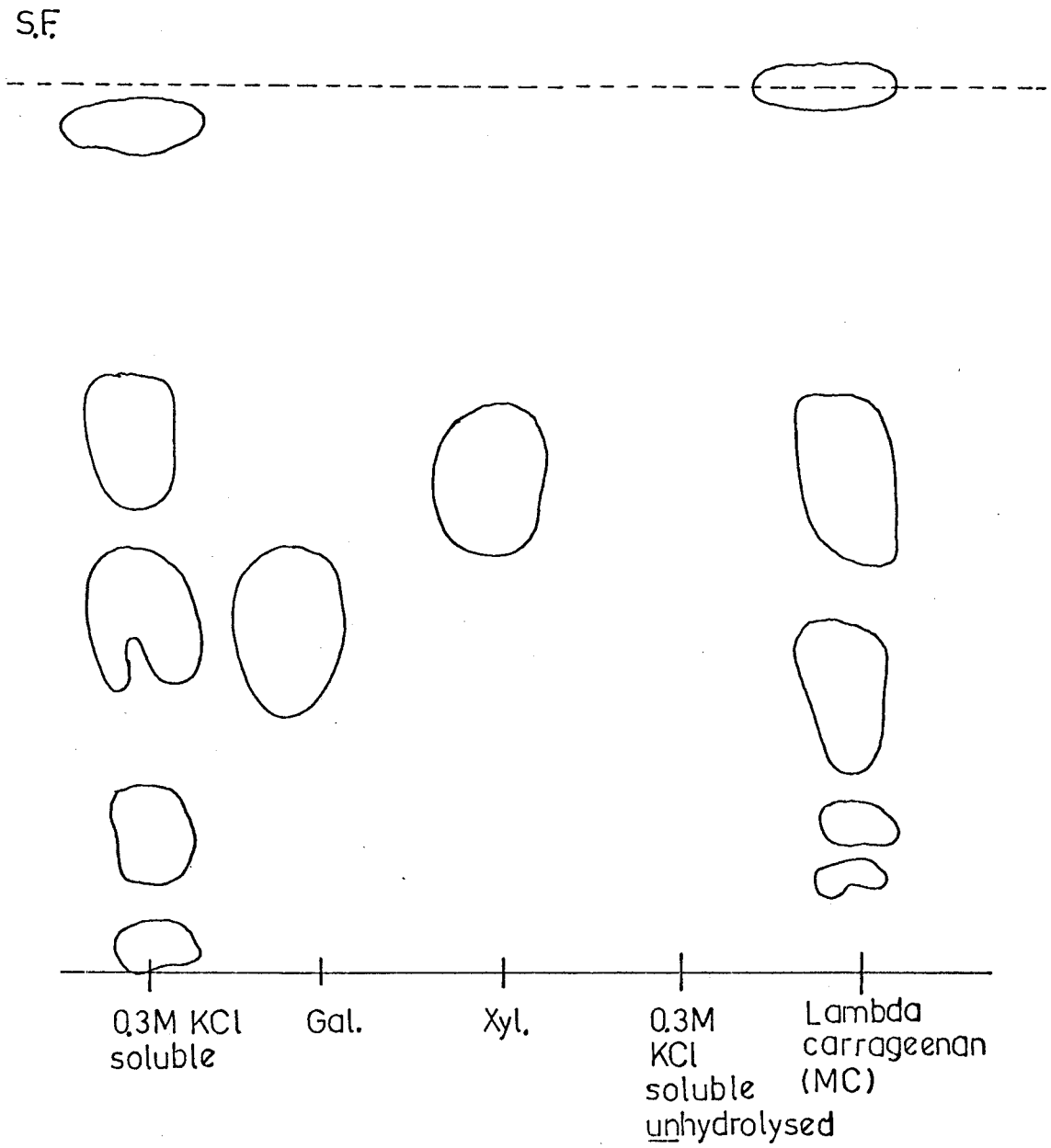

S.F.
0.3M KCl soluble
Gal.
Xyl.
0.3M KCl soluble unhydrolysed
Lambda carrageenan (MC)

Figure 9

Quantitative precipitin curve for γ-globulin from pre-injection serum and post-immunization serum (4 days after final injection).

(●——●) rabbit #1 - immune serum

(○——○) rabbit #2 - immune serum

(●- - -●) rabbit #1 - pre-immune serum

(○- - -○) rabbit #2 - pre-immune serum

Figure 9

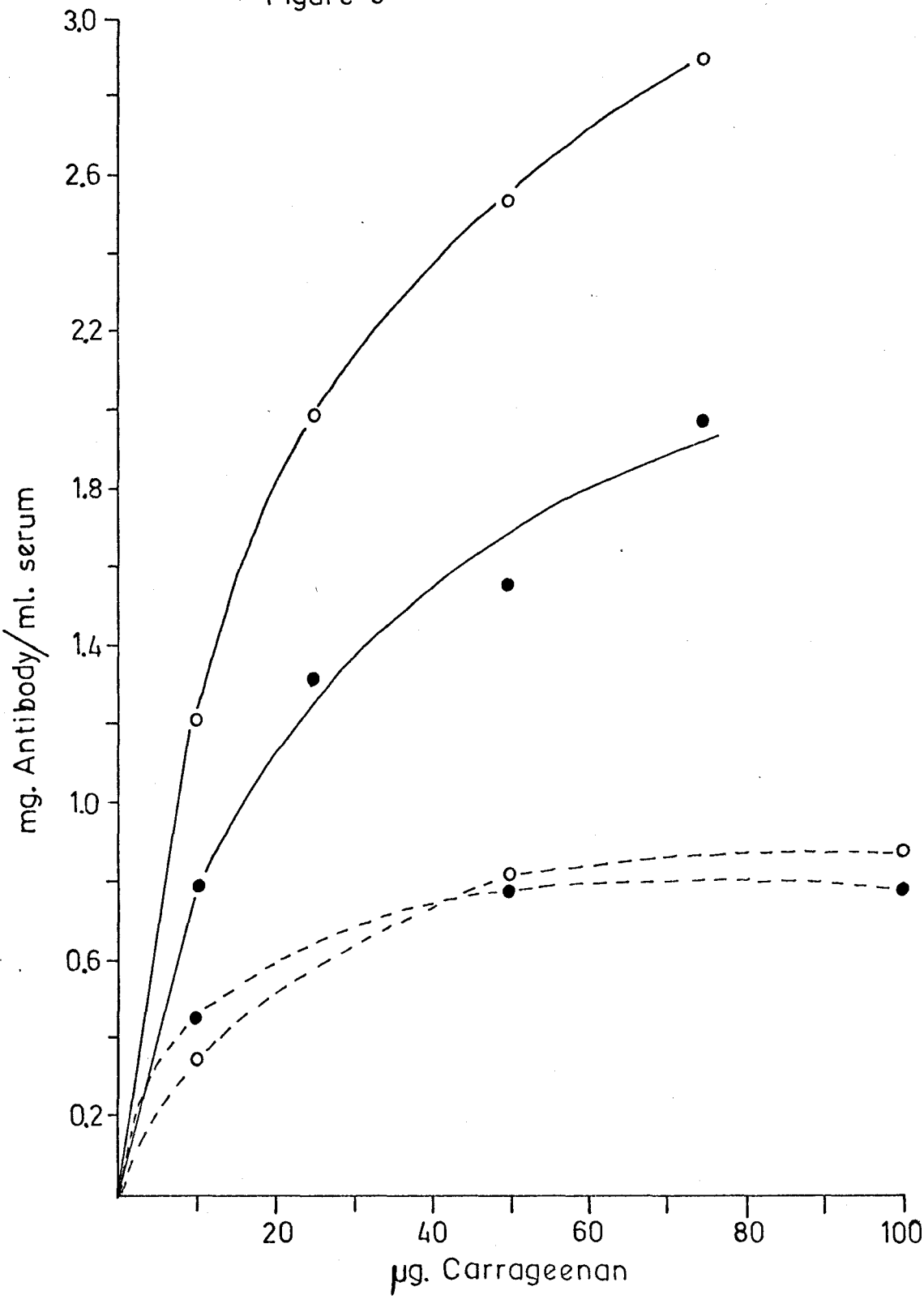
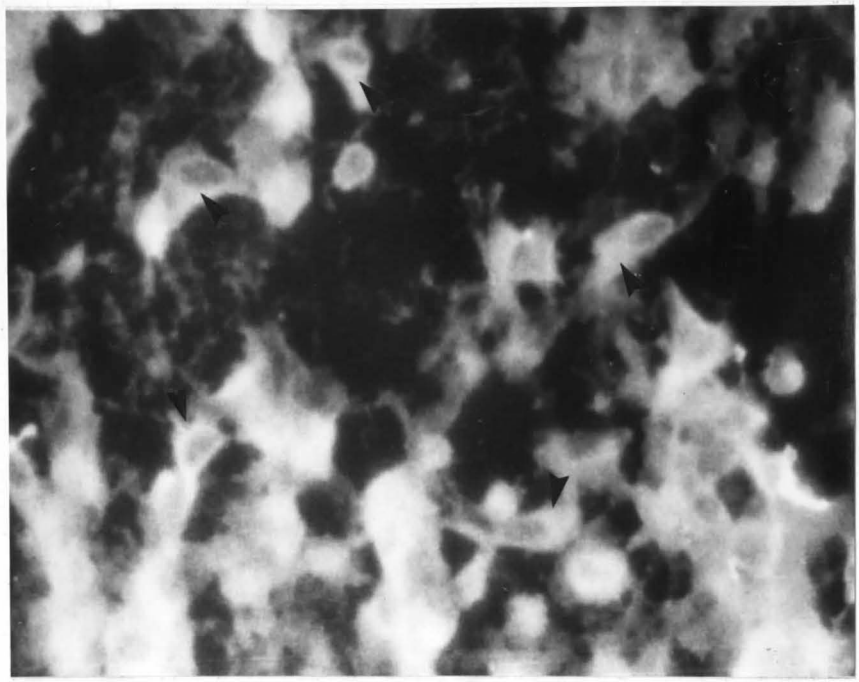

3.0
2.6
2.2
1.8
1.4
1.0
0.6
0.2
mg. Antibody/ml. serum
20
40
60
80
100
µg. Carrageenan

Figure 10

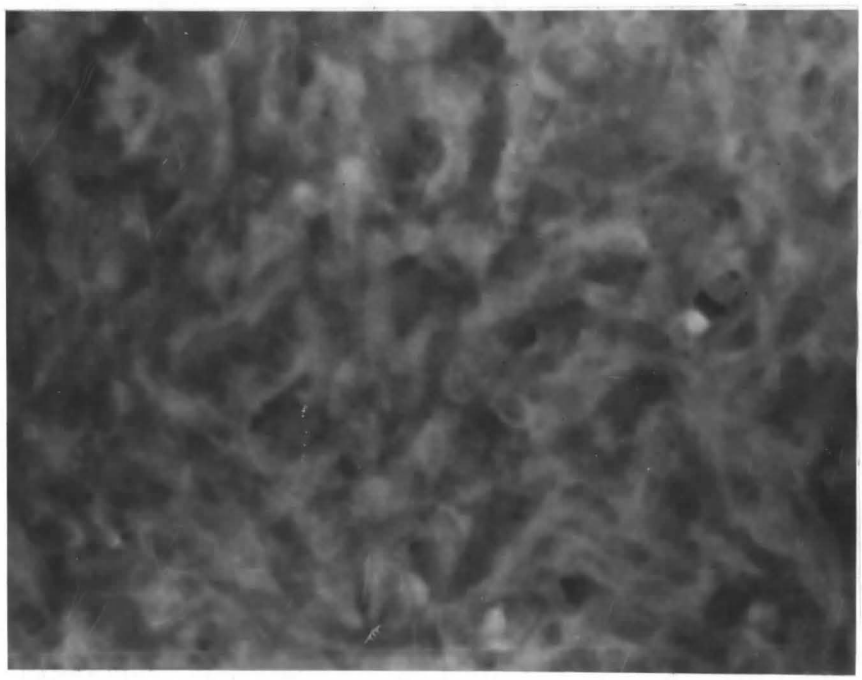
Section of granuloma, 7 days after injection of λ-carrageenan. Stained with undiluted fluorescent anti-λ-carrageenan antibody (30 min). Micrograph of slide observed with fluorescence microscopy. Arrows indicate the intracellular localization of the fluorescence. Compare with controls in Figures 11 and 12. X750

Figure 11

Section of granuloma, 7 days after injection of λ-carrageenan, cut from same tissue block as in Figure 10.
Control: stained with anti-λ-carrageenan antibody (30 min.), then with undiluted fluorescent anti-λ-carrageenan antibody (15 min). Micrograph of slide observed with fluorescence microscopy. This control shows no significant difference from Figure 12, which has not been exposed to labeled antibody.
X750



10



11

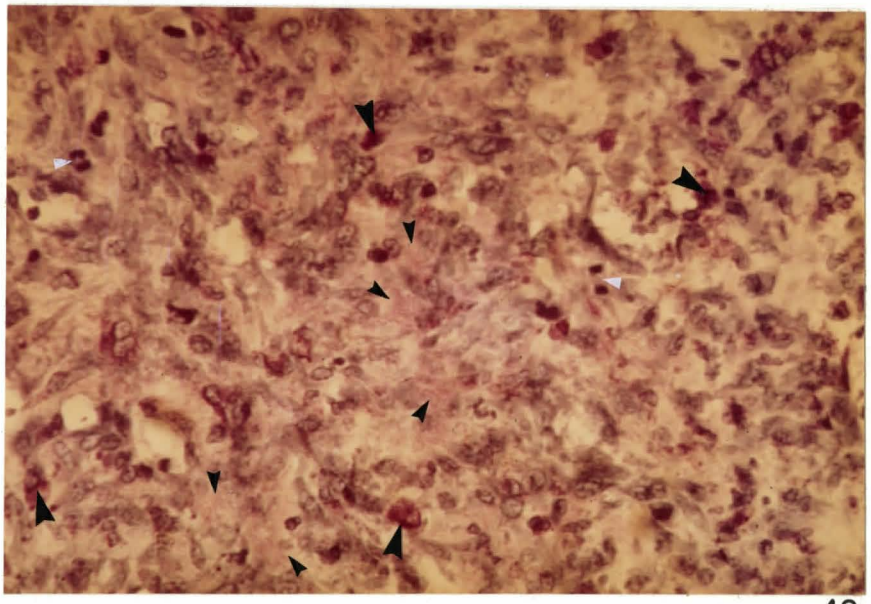
Figure 12

Section of granuloma, 7 days after injection of λ-carrageenan, cut from same tissue block as in Figures 10 and 11.
Control: section exposed to unlabeled anti-λ-carrageenan antibody (30 min). Micrograph of section observed with fluorescence microscopy. This is another control for Figure 10, and indicates the effectiveness of the control procedures used in Figure 11.
X750

Figure 13

Section of granuloma, 7 days after injection of λ-carrageenan. The section was cut from the same block of tissue as in Figures 10, 11, and 12. Micrograph of section stained with toluidine blue and observed with light microscopy. Note the extracellular metachromasia (small black arrows) and the darker staining intracellular metachromasia (large black arrows). Two mitotic figures are shown by the white arrows.
X420

12



13

Figure 14

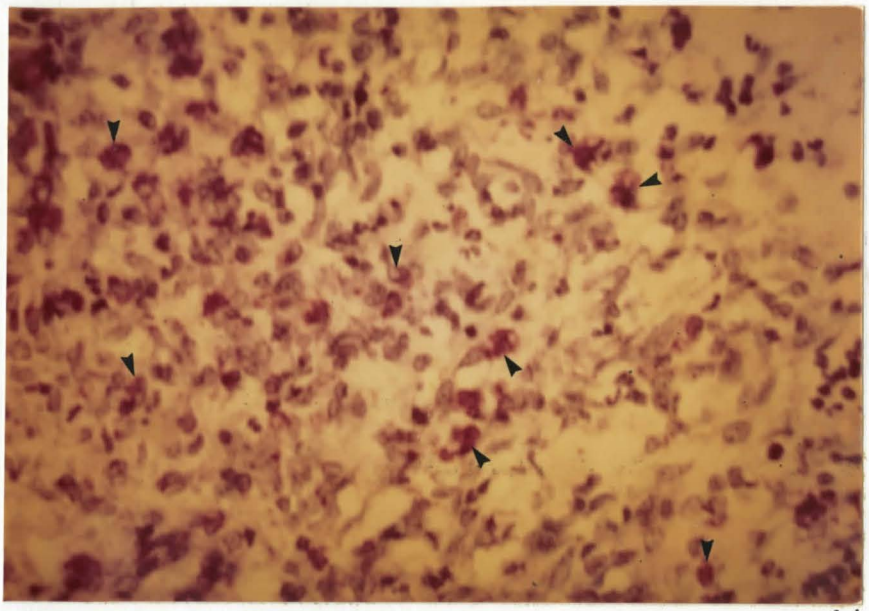
Section of granuloma, 7 days after injection of λ-carrageenan, section cut from same granuloma as in Figures 10 to 13. Micrograph of section stained with toluidine blue and observed with light microscopy. Note the cell associated metachromasia (arrows). Section taken from area of granuloma in which no extracellular metachromasia can be distinguished.

X420

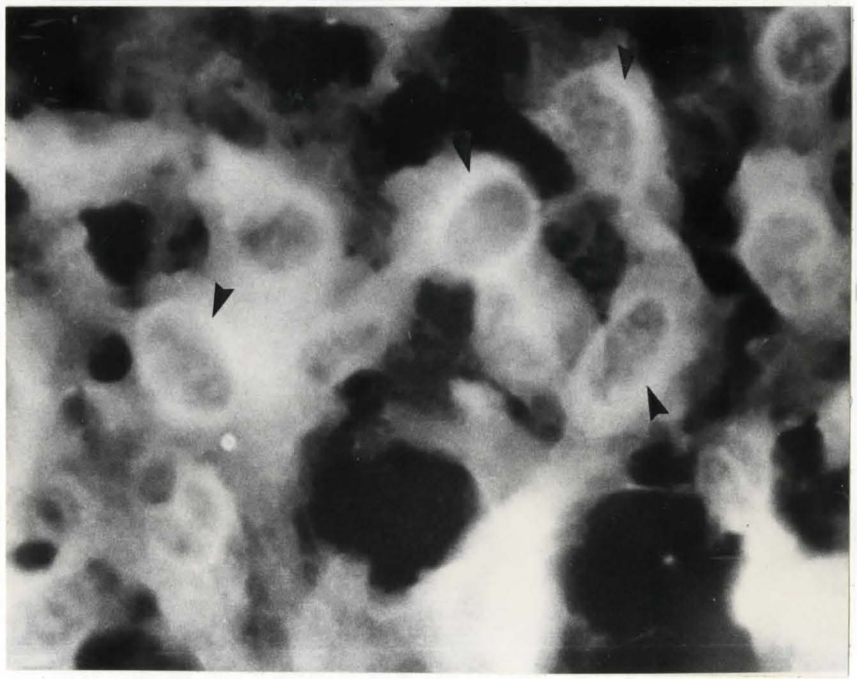
Figure 15

Section of granuloma, 7 days after injection of λ-carrageenan. Stained with undiluted fluorescent anti-λ-carrageenan antibody (30 min). Micrograph of section observed with fluorescence microscopy. Note intracellular fluorescence, especially intense in area surrounding the nucleus (arrows).

X1500



14



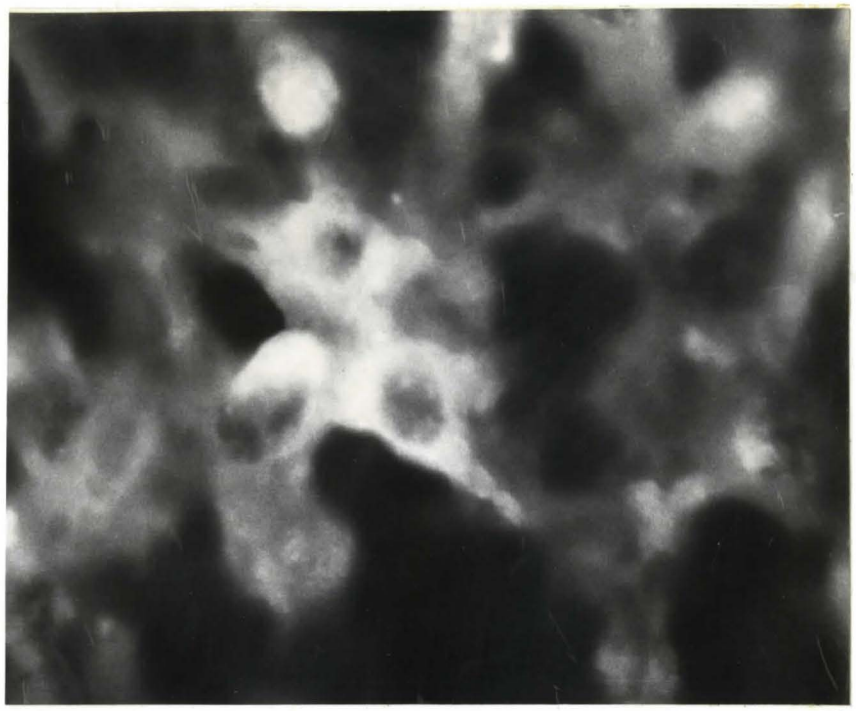
15

Figure 16

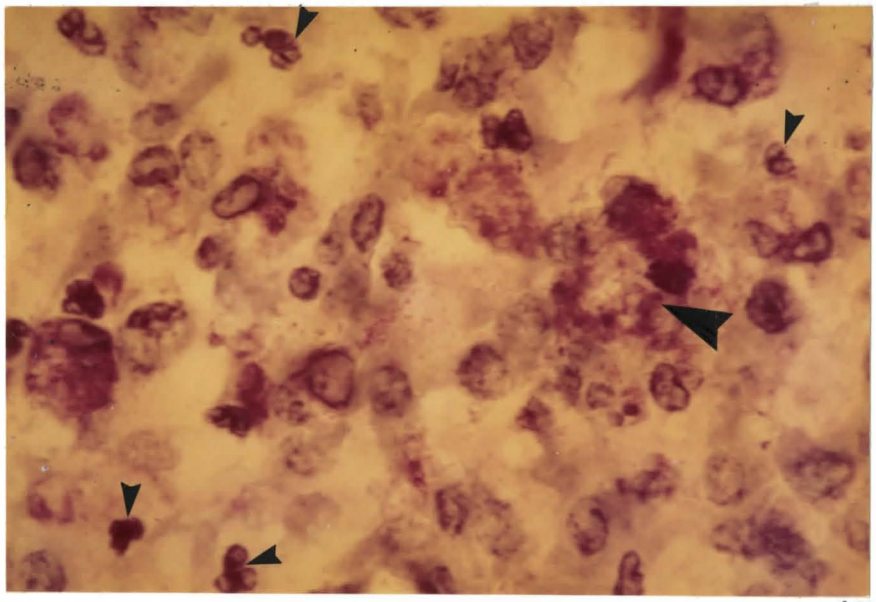
Section of granuloma, 7 days after injection of λ-carrageenan. Fluorescent anti-λ-carrageenan antibody stain diluted 1:2. Micrograph of section observed with fluorescence microscopy. Note intense fluorescence of a small cluster of cells.
X1500

Figure 17

Section of granuloma, 7 days after injection of λ-carrageenan. Micrograph of section stained with toluidine blue and observed with light microscopy. Note the presence of polymorphonuclear leukocytes (small arrows). The large arrow indicates an area where it is difficult to describe some of the metachromasia present as either intra or extracellular.
X920



16



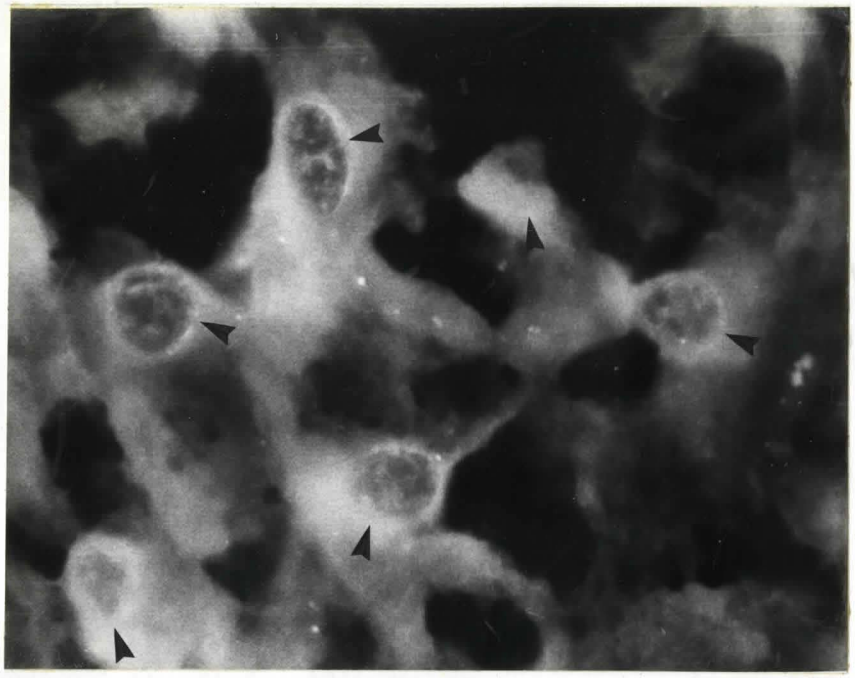
17

Figure 18

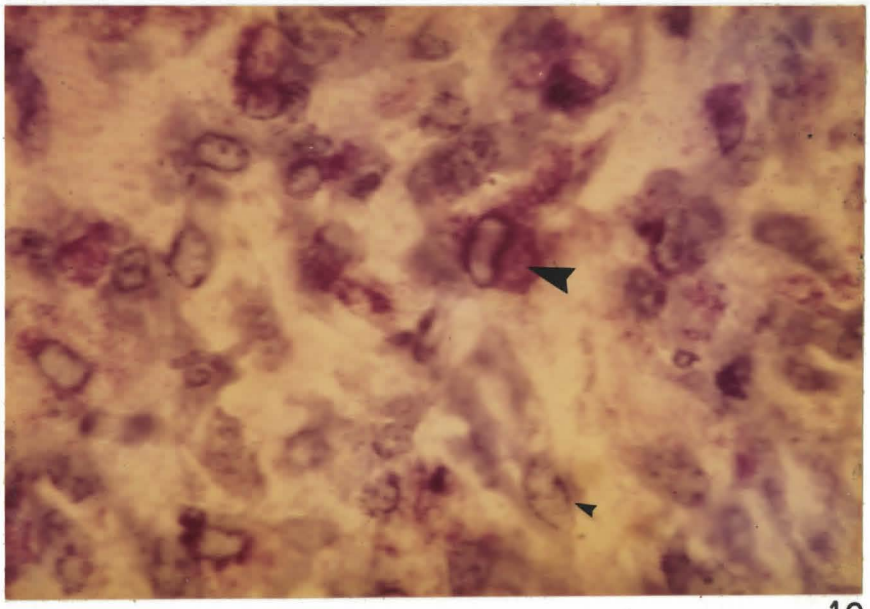
Section of granuloma, 7 days after injection of λ-carrageenan. Fluorescent anti-λ-carrageenan antibody diluted 1:2. Micrograph of section observed with fluorescence microscopy. Intense perinuclear staining (arrows) is more evident than in Figure 15.
X1500

Figure 19

Section of granuloma, 7 days after injection of λ-carrageenan. Micrograph of section stained with toluidine blue and observed with light microscopy. Note the macrophage (large arrow) with a large accumulation of metachromatic material around its kidney shaped nucleus. Also note a cell, interpreted as a fibroblast, which shows no intracellular metachromasia (small arrow).
X920



18



19

CHAPTER IV

DISCUSSION

A. Incorporation of ^{14}C into carrageenan

1. Yields

It should be noted that the amounts of carrageenan extracted and amounts of kappa and lambda carrageenan obtained by fractionation in the experiments reported here may not represent the true values to be found in seaweed in its natural habitat. This seaweed was kept in culture for various lengths of time (from 5 days to one month) before extraction. However, from the good physical appearance of the seaweed at extraction time, it seems reasonable to assume that extraction of "fresh" seaweed would give yields of the same order as those obtained in these experiments. The work by Black et al (1965) indicates several possible sources of variation. There would appear to be more variation due to locality than to the season of harvest, although this is not claimed by the author. In seaweed picked at one location, Black et al (1965) state that there is some evidence of a seasonal variation (summer weed gave a higher yield than autumn weed). However, in another set of samples from a different location there is no variation among summer, fall, winter or spring samples. Little can be concluded as to source of variation in amount of carrageenan extracted in the experiments reported in this thesis, since the seaweed was taken from different locations. However, the amount of carrageenan extracted from the different samples in each experiment is quite consistent.

Smith and Cook (1953) were the first to describe the fractional precipitation of carrageenan with KCl. They found about 40% of the carrageenan (extracted from Chondrus crispus) was precipitated at 0.125M

KCl; about 1% more was precipitated on raising the concentration to 0.25M KCl; this total fraction was designated K-carrageenan. About 45% of the carrageenan remained in solution; this fraction was designated λ-carrageenan. An extract of Chondrus crispus made at 60°C contained 64% kappa and 26% lambda carrageenan. The residue extracted at 100°C contained 14% kappa and 78% lambda carrageenan. When these data are summarized it would appear that Smith and Cook's total extract contained about equal amounts of kappa and lambda carrageenan. From the sharp fractionation obtained, Smith and Cook (1953) assumed these to be two distinct fractions. Later work has shown that both the purity of the fractions (in relation to the accepted structures for λ- and K-carrageenan as elucidated by Rees and his coworkers and discussed in the introduction of this thesis) and the amount recovered in each fraction is dependent on the KCl concentration as well as other factors.

The work of Pernas et al (1967) has shown the effect upon precipitation of varying the KCl concentration. Pernas et al (1967) found 48% of recovered carrageenan from Chondrus crispus was precipitated at 0.0625M KCl and 20% more was precipitated between 0.0625 and 0.25M KCl. Therefore, 68% was precipitated at 0.25M KCl; 31.4% was soluble at 0.25M KCl. Refractionation of the fraction soluble at 0.25M KCl at 1.5M KCl gave 16.3% soluble at 1.5M KCl and 15.1% insoluble at 1.5M KCl. According to the definition of Smith and Cook (1953), the 31.4% that was soluble is λ-carrageenan and the 68% precipitated at 0.25M KCl is K-carrageenan. The analysis for 3,6-anhydrogalactose of the different fractions obtained by Pernas et al (1967) was 29.2% 3,6-anhydrogalactose (3,6-AG) for the fraction precipitated at 0.0625M KCl, 27.0% 3,6-AG for that precipitated between 0.0625M and 0.25M KCl, 9.0% 3,6-AG for that soluble at 0.25M KCl.

The reprecipitation of the fraction soluble at 0.25M KCl at 1.5M KCl gave a soluble fraction with 4.0% 3,6-AG and an insoluble fraction with 15.0% 3,6-AG. These fractions thus all contain some kappa carrageenan and are not as pure as the λ-carrageenan reported in this thesis. The analysis for anhydrogalactose in the fraction to be designated as λ-carrageenan seems very important as an index of purity.

The fractionation procedure b) used in experiments #3 and #4 gave a very good separation. The lambda fraction recovered by this method is very pure (Stancioff, personal communication). The analysis of a lambda fraction obtained by fractionation method b), for 3,6-anhydrogalactose gave essentially no 3,6-anhydrogalactose, (McCandless and Richer, 1971). For this reason, significance is attached to the lambda carrageenan specific activity data discussed later.

A reason for the effectiveness of this method is that the precipitated kappa carrageenan is separated from the lambda carrageenan by filtration rather than by centrifugation. In method a), the fractions are separated by centrifugation. In our experience, this was not found to be as effective as filtration because of the gel nature of the precipitates; presumably the lambda fraction could contain some kappa carrageenan. Some of the gels are quite weak. It should be noted that, in the studies of both Smith and Cook (1953) and Pernas et al (1967), the two fractions were separated by centrifugation. The yield and especially the purity of the fractions are therefore dependent on the techniques used as well as the KCl concentration.

Black et al (1965) also studied the relative proportions of kappa and lambda carrageenan present in different samples of Chondrus crispus collected at different times and locations. The fractionations were done

at 0.25M KCl and separation done by centrifugation. Black et al (1965) found that fractionation into "pure" K and λ components was not always sharp. This effect appeared to be dependent on habitat. The content of 3,6-anhydrogalactose in the λ-carrageenan fraction varied from 2.2 to 10.1%. The K/λ ratio varied considerably with different samples and with the season of the year. Recent results from this laboratory suggest variation from sample to sample and possibly variation at different stages of the life cycle (unpublished data). It can be seen from the results that the proportion of λ-carrageenan obtained in the experiments reported here was quite low. This may be due in part to the purity of the fraction but also to the K/λ ratio of the particular plant used for extraction.

The analysis of the refractionation products of a kappa carrageenan, prepared by method b) and reported by McCandless and Richer (1971), indicated that an intermediate K_3 fraction probably contains a mixture of K-carrageenan, λ-carrageenan, and μ-carrageenan, therefore little can be said of this fraction. This fraction was relatively small (8.1 and 5.0% of total carrageenan before fractionation), so that it does not affect the yield of kappa carrageenan obtained in the first fractionation of carrageenan by method b, to any great extent.

2. Specific activity

The specific activity data in all four experiments indicate that λ-carrageenan is synthesized more rapidly than K-carrageenan. The specific activity data from experiment #2 show agreement with the results of Pernas et al (1967). The 3M KCl lambda carrageenan here may be compared to the fraction soluble at 1.5M KCl which Pernas et al, describe as highest in sulphate and lowest in 3,6-anhydrogalactose. This λ-carrageenan had the highest specific activity of any of the fractions obtained and

the lowest 3,6-anhydrogalactose content (1.3%). The refractionation of the 3M insoluble portion at 0.25M KCl gave a precipitable fraction of very low specific activity (3,6-AG content was 29%) and a 0.25M KCl soluble fraction with a specific activity intermediate between that of the 3M KCl soluble and the 0.25M KCl insoluble fractions. The 3,6-anhydrogalactose of this intermediate fraction was 16.5%. Pernas et al (1967) found this fraction, soluble at 0.25M KCl but precipitated at 1.5M KCl, to contain 15% 3,6-anhydrogalactose. The higher specific activity of the λ-carrageenan from experiment #2 when it was fractionated by method b) as compared to that obtained by method a) also supports the hypothesis of the higher purity of the λ-fraction claimed with method b). The 3M KCl λ-carrageenan from method a) represents a fraction relatively lower in specific activity, yet also very low in 3,6-anhydrogalactose. This suggests the presence of mu carrageenan rather than kappa carrageenan as a contaminant of this fraction.

The chromatographic separation of the hydrolysis products of the two lambda carrageenans, prepared in experiment #2, indicates the presence of a certain amount of xylose. This is in agreement with the work of Johnston and Percival (1950) who found, after hydrolysis of a carrageenan sample from Chondrus crispus with methanolic HCl at room temperature, a residue (15% of sample) with a low sulphate content which contained 70% galactose and 7% xylose. They stated this amount of xylose to be in agreement with the pentose content of the original polysaccharide. The radioactive analysis shows this xylose to contain ^{14}C. It is still not known if xylose is an integral part of the carrageenan structure. The other spot found corresponds to galactose as expected. The third spot found near the solvent front has not been identified; however it did not contain any significant radioactivity.

The analysis of the radioactive spots on the chromatograms indicates incomplete hydrolysis of the sample. The low recovery of activity from the chromatograms could be due to loss of sample during the preparation and staining of the chromatograms. The chromatogram presented in Figure 8 shows the similarity between the lambda carrageenan prepared in these experiments and the lambda carrageenan supplied by Marine Colloids, Inc., used as a reference sample. A sample from another labeling experiment done in this laboratory, prepared by method b), gave an infrared spectrum identical with this particular lambda carrageenan supplied by Marine Colloids (McCandless and Richer, 1971).

Experiment #3 and #4 included samples taken at different time intervals to give information on the rate of incorporation of ^{14}C into carrageenan. The variation of specific activity found in the λ-carrageenan seems to correlate with the light and dark periods and suggests a diurnal rhythm. This diurnal rhythm would seem to be endogenous as it was observed in both the experiments carried out under light/dark cycle (experiment #3) and continuous light conditions (experiment #4). This was also found in two additional experiments carried out under the same conditions (McCandless and Richer, 1971). In these experiments, the diurnal pattern of labeling persisted through two light/dark cycles. At longer exposure times, the pattern did not seem to be maintained. The diurnal pattern found was in accordance with the schedule of light under which the seaweed was maintained while in culture in our laboratory. The continuous light conditions did not give a higher rate of incorporation of ^{14}C into any of the carrageenan fractions.

Since the K_3 fraction is considered to be a mixture of λ, K and μ carrageenan, little meaning can be attributed to the specific activity of

this fraction other than that it was intermediate between the lambda and kappa fractions, as one might expect. The specific activity of the K_2 fraction (purer kappa) is not significantly different from the K_1 fraction since the amount of K_3 found was very small. Although there appeared to be a diurnal pattern in the specific activity of the K_1 fraction, it was not found consistently in the K_2 fraction of these experiments and the additional ones of McCandless and Richer (1971). The consistent appearance in K_1 may be due to contamination with a small amount of λ-carrageenan with a higher specific activity.

In experiments #3 and #4, the specific activity of the kappa fraction was only 3 to 5% of that of the lambda fraction. It was about 10% of the specific activity of the kappa fraction of other experiments (McCandless and Richer, 1971). The specific activity of the lambda fraction was also lower in experiments #3 and #4 than in the other experiments (about a 2-fold difference). The seaweed was collected at different times of the year and experiments #3 and #4 used winter weed, therefore one might suggest the difference was due to season. However, since appropriate data are not available, interpretation of the cause of variation is not possible until further work is done on the sources and magnitude of variation.

Few data are available on the mechanisms of biosynthesis of the carrageenans. Bean and Hassid (1954) investigated the intermediate products formed during photosynthesis with $^{14}CO_2$ by the red algae Iridophycus flaccidum, another carrageenan-producing red algae. The first steps of photosynthesis from CO_2 fixation to the formation of fluoroside (α-D-galactosyl-2-glycerol), which appears to be the main reserve product for the later assembly of the large polysaccharides, are studied in this paper. Bean and Hassid detected activity in the sulphurylated-galactan only after about one hour of photosynthesis. Bidwell (1958) surveyed

the rate of $^{14}CO_2$ assimilation and products of photosynthesis in various species of algae, including Chondrus crispus. He found the main product to be fluoroside but stated that its route of formation was not yet clearly understood.

No data, however, are available on the later mechanisms of synthesis of the larger polysaccharides, the carrageenans.

Certain aspects of the biosynthesis of the carrageenans can be elucidated from the experiments reported here and by McCandless and Richer (1971). Lambda carrageenan seems to be synthesized more rapidly than kappa carrageenan. Also the specific activity time curves do not suggest that lambda is an immediate precursor of kappa carrageenan (Zilversmit et al, 1943). Our results agree with Rees (1963), who proposes μ-carrageenan as a precursor of kappa carrageenan. McCandless and Richer (1971) report an analysis of a ^{14}C λ-carrageenan (extracted after 24 hours of ^{14}C exposure plus a 2 hour chase) prepared in a labeling experiment similar to those reported here. After passing the sample through DEAE Sephadex A50, they found the highest sulphated polymers had a low specific activity. They suggest that this indicates that complete sulphation occurs after ^{14}C is introduced into the molecule. An analysis on agarose showed that at this time (24 hours of photosynthesis) the largest polymers were not labeled (McCandless and Richer, 1971). Further ^{14}C incorporation studies and analyses of this sort should help in the elucidation of the biosynthetic pathways of the carrageenans.

B. Localization of lambda carrageenan in the granuloma

1. Fluorescent antibody

(a) General considerations

The importance of the development of fluorescent antibodies as a histological stain lies in the extreme specificity that each antibody has for its homologous antigen. Such a specificity is required to identify conclusively a particular compound in a histological section which contains other substances chemically related to the compound in question. Such is the case with the carrageenan granuloma. The positive identification of lambda carrageenan within the connective tissue granuloma is impossible with the usual histochemical methods due to the presence of the naturally occurring mucopolysaccharides of the connective tissue matrix. The similarity of carrageenan to chondroitin sulphate has led to the suggestion that lambda carrageenan may bring about the stimulation of connective tissue by substituting for the naturally occurring mucopolysaccharides (McCandless, 1965).

The strong metachromatic reaction of carrageenan with toluidine blue has led to the extensive use of this dye in studying the granuloma. The toluidine blue is used at pH 3-3.5, since at this pH the carboxyl groups are not ionized and the metachromasia is caused by the presence of ionized sulphate groups. However, this stain is not specific for carrageenan since metachromasia will also result from certain mucopolysaccharides of the connective tissue matrix. Indirect proof that some of the metachromasia is due to carrageenan has been given by treating the tissue sections with testicular hyaluronidase prior to staining with toluidine blue (Williams, 1957). By this treatment, metachromasia of

the ground substance fails to appear whereas the metachromasia of the carrageenan is said not to be affected.

Johnston (1968) characterized the nature of the carrageenan anti-carrageenan antibody system in the rabbit and suggested the possible use of fluorescent antibody to identify and localize carrageenan in the granuloma. Johnston (1968), in cross reaction studies with anti-λ-carrageenan antibody and related compounds, showed that there was no precipitation of anti-λ-carrageenan antibody by chondroitin sulphate. The successful use of the fluorescent antibody techniques is dependent on the antigen's being present in its antigenic state. If the antigen were altered by the metabolizing processes of the tissue, its identification with fluorescent antibody would be impossible.

(b) Localization of λ-carrageenan in the granuloma

The main difficulty in using fluorescent antibody techniques with the lambda carrageenan granuloma was the greenish autofluorescence which could not be eliminated and which hindered interpretation of positive results. A two step inhibition procedure was used to obtain controls, whereby the staining reaction is blocked by the previous exposure of the sections to unlabeled antibody, since the antigenic sites have become occupied with unlabeled antibody. When the labeled antibody is applied to the tissue, the antigen-antibody complex is in equilibrium with excess antibody and some replacement by labeled molecules may occur (Kawamura, 1969). For this reason, the tissue is exposed to the labeled antibody for a shorter period of time. If significant staining of the sections is present in the controls, it can then be attributed to a non-specific precipitation of the antibody. The results from the controls in these experiments plus the lack of improvement in staining when the antibody was absorbed with tissue

powder indicated that the fluorescent antibody preparation used was specific. Another control was provided by applying unlabeled antibody to the sections. The faint fluorescence of these slides is due to the autofluorescence of the tissue.

The results indicate that lambda carrageenan is present in its antigenic state within the cytoplasm of the cells of the granuloma. It was not possible at this time to limit the cell type which exhibited fluorescence since difficulty was noted in differentiating fibroblasts and macrophages in the granuloma when the tissue was observed for fluorescent antibody. It was not possible to differentiate between these two cell types in sections stained with haematoxylin and eosin either. Williams (1957) also noted this difficulty in sections stained with haematoxylin and eosin. He used the metalophil staining properties as well as the "metachromasia" of their ingested carrageenan to identify macrophages.

The fibroblast is active in the production of collagen, which is considered its main role in the formation of connective tissue. The macrophage on the other hand has its main role in the phagocytosis of particulate matter. Little phagocytosing ability is attributed to the fibroblast. The latter characteristic has been used to differentiate macrophages and fibroblasts in connective tissue since, after injection of a vital dye such as trypan blue, the dye can be seen concentrated in the cytoplasm of macrophages and little is seen in the fibroblasts. However, little morphological distinction can be made between the two cell types after staining with a general stain such as haematoxylin and eosin. It would be expected that carrageenan appear in the cytoplasm of macrophages because of their phagocytosing ability: however, the

possibility of carrageenan being present in the cytoplasm of fibroblasts can not be excluded. Pérez-Tamayo (1970) in an electron microscope study of the carrageenan granuloma in guinea pigs reported the presence of "homogeneous, structureless electron dense globules" in the cytoplasm of both macrophages and fibroblasts. These "hyaline globules" were interpreted as carrageenan.

The absence of extracellular carrageenan in the young granuloma (about 7 days) would seem unreasonable in view of the amount of carrageenan injected into what can be considered a very localized area. In the toluidine blue stained sections, the prominent extracellular metachromatic material in the young granuloma indicates the probability of extracellular carrageenan. Williams (1957) reported that by the tenth day much of the extracellular carrageenan had disappeared. However, Pérez-Tamayo (1970) reported that after 7 days no extracellular homogeneous, amorphous and electron dense material was found. The fluorescent antibody studies of the 7 day granuloma presented in this thesis seem to agree with Pérez-Tamayo. However, certain explanations could be given to explain the failure of fluorescent antibody to confirm the presence of extracellular carrageenan. Macrophages, when phagocytizing a foreign substance, concentrate this substance in their cytoplasm. When the cells accumulate carrageenan in their cytoplasm, the concentration is perhaps high enough to give the very bright fluorescence observed which is easily distinguishable from that of the controls. The fluorescence of the extracellular spaces may not appear significant as compared to the controls. Another explanation could be that the extracellular carrageenan is not immunologically reactive. Lambda carrageenan is known to bind to proteins (Turner and Magnusson, 1962). It is reasonable to assume, therefore,

that λ-carrageenan when injected associates with the proteins present and perhaps in this state is not antigenic. No evidence is available, however, to support this statement. The lambda carrageenan in the cell cytoplasm must be in its antigenic state since a positive reaction was obtained.

(c) Staining of other tissues

When an antigen is inoculated into tissues (e.g. skin, subcutaneous tissue, muscle), it is carried through the lymphatics to neighboring lymph nodes where antibody production is initiated. Similarly it can be carried to the spleen (Davis et al, 1968). The hypersensitive state caused in guinea pigs by the previous injection of carrageenan (McCandless, 1967) suggests an immunological response. For these reasons the spleen and parietal lymph nodes were taken for investigation with fluorescent antibody. Staining of these tissues did not give any positive results. It should be noted, however, that carrageenan has not been shown to be antigenic in guinea pigs. Prior attempts to demonstrate antibodies to λ-carrageenan in guinea pig serum of animals injected subcutaneously with λ-carrageenan in this laboratory were not successful (unpublished data).

The fluorescent antibody studies were terminated because a mechanical failure in the deep freeze allowed the stored fluorescent antibody to thaw. The consequent denaturation of the preparation prevented its further use. Also, by this time the original strain of rabbits used by Johnston (1968), and in the production of antibodies used for the fluorescent labeling in the studies reported here, was no longer available. Subsequently, all other rabbits tried failed to produce specific antibody to any of the carrageenan fractions. Work by Krause (1970) shows that the ability of rabbits to produce antibody to polysaccharides

is genetically determined. This suggests that the ability of the first strain of rabbits and the unresponsiveness of the subsequent rabbits may have been genetically determined. The immunogenicity of carrageenan in other species is presently being investigated. Further work with fluorescent antibody and carrageenan localization will be possible when specific antibody to the carrageenan fractions is available.

(d) Peritoneal cell staining

These experiments were done after the failure of the deep freeze had allowed the stored antibody to thaw. Due to the denaturation of the antibody preparation, the results are uninterpretable. These experiments are mentioned only to indicate the possibility of testing the specificity of the antibody solution for the particular carrageenan fraction by providing a system less complex than the granulation tissue.

2. Autoradiography

Autoradiography of ^{14}C-λ-carrageenan granulomas was done to confirm and supplement the localization obtained with the fluorescent antibody. The techniques and principles involved in autoradiographic localization are much simpler than those used in fluorescent antibody studies. In this sense, autoradiography should provide a good comparison for the results obtained with fluorescent antibody.

The negative results obtained with autoradiography can be explained by the fact that the ^{14}C labeled λ-carrageenan available did not have a high enough specific activity to produce an autoradiographic image. The highest specific activity λ-carrageenan injected was 27.6 dpm/μg galactose. The total activity administered to the guinea pig was 0.18 μc. Although it is difficult to estimate exposure times, Rogers (1967) gives a method of estimating the length of exposure needed. Rogers (1967) states that, assuming even distribution of the isotope in the animal, if 1 μc/g (of animal) is injected, 1000 μ^3 of tissue will give approximately 3.2 disintegrations/day. Also, for ^{14}C, a section 5 μ thick and nuclear emulsion 2-4μ thick, 100 disintegrations will give 30-40 silver grains. Even if we assume that the lambda carrageenan is localized at the site of injection, the concentration of activity is very low. The exposure time required would be too long and consequently the background level too high to give meaningful results. Exposures of up to 5 weeks were tried but by this time background was too high.

It was found in the metabolism experiments that some of the activity injected can be reccvered in the urine within the first 24 hours after injection. At this time 16 and 19% of the activity was recovered from 2 of the 3 guinea pigs tested for excretion of ^{14}C. Failure to detect

^{14}C in the urine of one of the guinea pigs could be due to dilution of the ^{14}C. The appearance of ^{14}C in the urine such a short time after injection and the absence of any further activity at later times may be due to the presence of small (excretable) molecules in the carrageenan solution, rather than due to the animals metabolizing carrageenan. Though the solution is dialysed, which should remove small molecules, it is subsequently autoclaved before administration. This can cause a certain amount of hydrolysis of the carrageenan (a drop in viscosity is noted after autoclaving) resulting in the presence of small molecules.

Although these autoradiography results are negative, positive results should be possible when lambda carrageenan of sufficiently high specific activity is available. The autoradiography should give a direct correlation with the metachromasia as the sections can be stained with toluidine blue after radiographic development.

SUMMARY

The studies on the incorporation of ^{14}C into the different carrageenan fractions extracted from Chondrus crispus show that the label is more rapidly introduced into λ-carrageenan than K-carrageenan.

A diurnal pattern of labeling was observed in λ-carrageenan, up to 48 hours of ^{14}C exposure, which corresponded to the light/dark schedule of culture. The rhythm seems to be endogenous as it was also observed in seaweed exposed to ^{14}C under continuous light conditions. The diurnal pattern was not consistent in the kappa carrageenan.

The specific activity time curves do not suggest that lambda carrageenan is an immediate precursor of kappa carrageenan.

Sources of variation in yields of the different carrageenan fractions and of specific activity in these and other reported experiments are not yet fully understood.

Lambda carrageenan has been localized in the cytoplasm of the cells of the carrageenan granuloma in the guinea pig by fluorescent antibody techniques.

The presence of extracellular carrageenan in the granulomas studied was not confirmed with fluorescent antibody.

REFERENCES

Allison, B. M., Harrington, J. S. and Birbeck, M. 1966: An examination of the cytotoxic effects of silica on macrophages. J. Exp. Med., 124: 141-153

Anderson, N. S., Dolan, T. C. S., Penman, A., Rees, D. A., Mueller, G. P., Stancioff, D. J. and Stanley, N. F. 1968: Carrageenans. Part IV. Variation in the structure and gel properties of K-carrageenan, and the characterisation of sulphate esters by infrared spectroscopy. J. Chem. Soc. (C),: 602-606

Bean, R. C. and Hassid, W. Z. 1955: Assimilation of $C^{14}O_2$ by a photosynthesizing red alga, Iridophycus flaccidum. J. Biol. Chem., 212: 411-425

Benitz, K. F. and Hall, L. M. 1959: Local morphological response following a single subcutaneous injection of carrageenin in the rat. Proc. Soc. Exper. Biol. Med., 102: 442-445

Bidwell, R. G. S. 1958: Photosynthesis and metabolism of marine algae. II. A survey of rates and products of photosynthesis in $^{14}CO_2$. Can. J. Bot., 36: 337-349

Black, W. A. P., Blakemore, W. R., Colquhoun, J. A. and Dewar, E. T. 1965: The evaluation of some red algae as a source of carrageenan and its K and λ-components. J. Sci. F. Agric., 16: 573-585

Cherry, W. B., Goldman, M., and Carski, T. R. 1960: Fluorescent antibody techniques in the diagnosis of communicable diseases. U.S. Public Health Serv. Publ. 729

Curtain, C. C. 1961: The chromatographic purification of fluorescent antibody. J. Histochem. Cytochem., 9: 484-486

Davis, D. D., Dulbecco, R., Eisen, H. N., Ginsberg, H. S., Wood Jr., W. B.: Principles of microbiology and immunology. Harper and Row, Publishers, Inc., New York, 1968

Dolan, T. C. S. 1965: The structure and biosynthesis of polysaccharides in the red seaweeds. Ph.D. Thesis, Edinburgh

Dolan, T. C. S. and Rees, D. A. 1965: The carrageenans. Part II. The positions of the glycosidic linkages and sulphate esters in λ-carrageenan. J. Chem. Soc., 649: 3534-3539

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, H. 1956: Colorimetric method for determination of sugars and related substances. Analyt. Chem., 28: 350-356

Fisher, E. R. and Parr, J. 1960: Carrageenin granuloma in the guinea pig and rat. I. Effect of hydrocortisone, estradiol and mast cell depletion on its histological and histochemical features. Arch. Path., 70: 565-575

Fritsch, T. E.: The Structure and Reproduction of the Algae. Cambridge, 1945.

Goldman, M.: Fluorescent Antibody Methods. Academic Press, Inc., New York, 1968

Gude, W. D.: Autoradiographic Techniques. Localization of Radioisotopes in Biological Material. Prentice-Hall, Inc., New Jersey, 1968

Hamerman, D. 1955: Staining methods in chromatography of acidic and neutral mucopolysaccharides. Science, 122: 924

Heidelberger, M. and MacPherson, C. P. C. 1943: Quantitative microestimation of antibodies in the sera of man and other animals. Science, 97: 405-406

Hellebust, J. A. and Haug, A. 1969: Alginic acid synthesis in Laminaria digitata (L) Lamour. In: Proc. Sixth Intern. Seaweed Symp., 1968. Edited by R. Margalef. Madrid. p463-471

Humason, G. L.: Animal Tissue Techniques. W. H. Freeman and Company, San Francisco, 1962

Jackson, D. S. 1956a: The stimulation of connective tissue formation by carrageenin. Biochem. J., 62: 25

_____ 1956b: The formation and removal of collagen in the carrageenan granuloma. Biochem. J., 64: 8

_____ 1957: Connective tissue growth stimulated by carrageenin. I. The formation and removal of collagen. Biochem. J., 65: 277-284

Johnston, K. H. 1968: The immunological response in rabbits to carrageenans, sulphated galactans extracted from marine algae. M.Sc. thesis, McMaster University

Johnston, K. H. and McCandless, E. L. 1967: Immunological response to carrageenan. Fed. Proc., 26: 703

_____ 1968: The immunologic response of rabbits to carrageenans, sulphated galactans extracted from marine algae. J. Immunol., 101: 556-562

Johnston, R. and Percival, E. 1950: The polysaccharides of carragheen. Part III. Confirmation of the 1:3-linkage in carrageenin, and the isolation of L-galactose derivatives from a resistant fragment. J. Chem. Soc.: 1994-1998

Kawamura Jr., A., ed.: Fluorescent Antibody Techniques and Their Applications. University of Tokyo Press, Tokyo, 1969

Lillie, R. D.: Histopathologic Technique and Practical Histochemistry. Blakiston Co., Inc., Philadelphia, 1954

Lowry, O. H., Rosenborough, N. J., Farr, L. and Randall, R. J. 1951: Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275

McCandless, E. L. 1965: Chemical structural requirements for stimulation of connective tissue growth by polysaccharides. Ann. N. Y. Acad. Sci., 118: 867-882

_____ 1967: Sensitization reaction to carrageenan in the guinea pig. Proc. Soc. Exp. Biol. Med., 124: 1239-1242

_____ and Lehoczky-Mona, J. 1964: Identification of the active fraction of carrageenan in stimulation of connective tissue growth. Growth, 28: 143-156

_____ 1966: Connective tissue stimulation in guinea pigs by polysaccharides derived from marine algae. In: Proc. Fifth Intern. Seaweed Symp., 1965. Edited by E. G. Young and J. L. McLachlan. Permagon Press, London. p.157-165

_____ and Richer, S. 1971: ^{14}C studies on carrageenan synthesis. In: Proc. Seventh Inter. Seaweed Symp. 1971. In Press

Monis, B., Weinberg, T. and Spector, G. J. 1968: The carrageenan granuloma in the rat, a model for the study of the structure and function of macrophages. British J. Exp. Path., 49: 302-310

O'Neill, A. N. 1955: 3,6-Anhydro-D-galactose as a constituent of K-carrageenan. J. Am. Chem. Soc., 77: 2837-2839

_____ 1955: Derivatives of 4-O-B-D-galactopyranosyl-3,6-anhydro-D-galactose from K-carrageenan. J. Am. Chem. Soc., 77: 6324-6326

Painter, T. J. 1966: The location of the sulphate half ester groups in furcellaran and K-carrageenan. In: Proc. Fifth Intern. Seaweed Symp., 1965. Edited by E. G. Young and J. L. McLachlan. Permagon Press, London. p.305-313

Percival, E. and McDowell, R. H.: Chemistry and Enzymology of Marine Algal Polysaccharides. Academic Press, Inc., New York, 1967

Pérez-Tamayo, R. 1970: Collagen resorption in carrageenin granulomas. I. Collaginic activity in in vitro explants. II. Ultrastructure of collagen resorption. Lab. Invest., 22: 137-159

Pernas, A. J., Smidsrod, O., Larson, B. and Haug, A. 1967: Chemical heterogeneity of carrageenans as shown by fractional precipitation with potassium chloride. Acta. Chem. Scand., 21: 98-110

Prescott, D. M., ed.: Methods in Cell Physiology V.1. Academic Press, Inc., New York, 1964

Pridham, J. B. 1956: Determination of sugars on paper chromatograms with p-anisidine hydrochloride. Analyt. Chem., 28: 1967-1968

Rees, D. A. 1960: Paper chromatography of acidic polysaccharides. Nature, 185: 309-310

_____ 1963: The carrageenan system of polysaccharides. Part I. The relation between K and λ components. J. Chem. Soc.,: 1821-1832

_____ 1965: Carbohydrate sulphates. Chem. Soc. Ann. Rep. 62: 469-487

Robertson, W. van B. and Schwartz, R. 1953: Ascorbic acid and the formation of collagen. J. Biol. Chem., 201: 689-696

Rogers, A. W.: Techniques in Autoradiography. Elsevie Publishing Co., New York, 1967

Rosenau, W., Moon, H. D. and McIvor, B. C. 1962: Localization of antibody to tissue culture cells by fluorescent antibody techniques. Lab. Invest., 11: 199-203

Sainte-Marie, G. 1962: A paraffin embedding technique for studies employing immunofluorescence. J. Histochem. Cytochem., 10: 250-256

Slack, H. G. B. 1957: Connective tissue growth stimulated by carrageenin. 2. Metabolism of sulphated polysaccharides. Biochem. J., 65: 459-464

Smith, D. B. and Cook, W. H. 1953: Fractionation of carrageenin. Arch. Biochem. Biophys., 45: 232-233

_____ and Neal, J. L. 1954: Physical studies on carrageenin and carrageenin fractions. Arch. Biochem. Biophys., 53: 192-204

Somogyi, M. 1952: Notes on sugar determination. J. Biol. Chem., 195: 19-23

Stancioff, D. J. (to Marine Colloids, Inc.) 1965: Selective extraction of hydrocolloid fractions from sea plants. U.S. Pat. 3, 176, 003

Stassen, F. L. H. 1970: Connective tissue reactions to carrageenin, a combined morphological and biochemical study. Ph.D. thesis. Catholic University of Nijmegen

Turner, K. J. and Magnusson, B. J. 1962: Carrageenin as a protein precipitant. Nature, 194: 451-452

Vreeland, V. 1970: Localization of a cell wall polysaccharide in a brown algae with labelled antibody. J. Histochem. Cytochem., 18: 371-373

_____ 1971: An immunological study of extracellular polysaccharides in the brown algae, Fucus distichus. Ph.D. thesis, Stanford University

Weigl, J., Turvey, J. R. and Yaphe, W. 1966: The enzymatic hydrolysis of K-carrageenan with K-carrageenase from Pseudomonas carrageenovora. In: Proc. Fifth Intern. Seaweed Symp., 1965. Edited by E. G. Young and J. L. McLachlan. Pergamon Press, London. p.329-332

Weir, D. M., ed.: Handbook of Experimental Immunology. F. A. Davies Company, Philadelphia, 1967

Williams, G. 1957: A histological study of the connective tissue reaction to carrageenin. J. Path. Bact., 73: 557-563

Yaphe, W. and Arsenault, G. P. 1965: Improved resorcinol reagent for the determination of fructose and of 3,6-anhydrogalactose in polysaccharides. Analyt. Biochem., 13: 143-148

Zilversmit, D. B., Entenman, O., and Fishler, M. C. 1943: On the calculation of "turnover time" and "turnover rate" from experiments involving the use of labelling agents. J. Gen. Physiol., 26: 325-340