AN IMMUNOCHEMICAL STUDY OF CARRAGEENANS IN GAMETOPHYTE AND SPOROPHYTE GENERATIONS OF CERTAIN RED ALGAE

by

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Immunochemistry of Carrageenans

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SCOPE AND CONTENTS:

Carrageenans are a heterogenous population of sulphated galactans extracted from certain marine algae. Fractionation of these polysaccharides with potassium chloride yields an insoluble fraction designated κ- carrageenan and a soluble fraction designated λ- carrageenan. Recent evidence has shown that certain algae in the gametophytic stage of the life cycle produced κ- carrageenan and these algae in the sporophytic stage produced λ- carrageenan. The present study was undertaken to confirm this observation by using immunochemical techniques. Specific antibodies against κ- and λ- carrageenan were produced in goats and these were used to determine the "kappa like" and "lambda like" properties of carrageenans from the different stages of red algae.

PREFACE

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INTRODUCTION

The Rhodophyta

According to classical taxonomic classification based on reproductive structures and plant morphology, the Rhodophyta (red algae) is one of several algae divisions (Kylin, 1956). It has one class, the Rhodophyceae. The subclass Florideae has been divided into six orders. Only algal species of the order Gigartinales will be considered in this thesis. Gigartinales has been divided into six families: Furcellariaceae, Solieriaceae, Hypneaceae, Gracilariaceae, Phyllophoraceae and Gigartinaceae.

Widespread interest has developed in algal species of Gigartinales due to their production of the commercially important gelans, agars, and carrageenans (Stoloff and Silva, 1957). The enzymatic classification of Yaphe (1959) has revealed that most species of Gigartinales produce carrageenan, some produce agar and a few species outside this order produce carrageenans. This discrepancy has prompted some investigators (Stoloff, 1962) to propose that the algae be reclassified with emphasis being placed on the structural polysaccharides they contain.

Chondrus Crispus

Chondrus crispus, or as it is more commonly known, Irish Moss, is a species of red algae classified under the family Gigartinaceae in the order Gigartinales. It is common on the North Atlantic coasts and is one of the primary producers of carrageenan.

The life history of C. crispus has been demonstrated completely in culture (Chen and McLachlan, 1972). There is a sequence of isomorphic plants consisting of male and female gametophytes, tetrasporophytes, and carposporophytes. Tetraspores released from the sporangia of the tetrasporic plant germinate to give rise to gametophytic plants. When the male plant matures it produces male gametes which are released as spermatidia. These presumably fertilize the female plant and initiate the development of carpospores. Carpospores are eventually released and upon germination give rise to tetrasporic plants.

Carrageenans

Carrageenans are a mixture of sulphated galactans extractable with hot water. Commercially they are important because of their gelling properties (Rose and Cook, 1949). It has been shown that the gel strength can be increased by addition of potassium chloride (Reedman and Buckby, 1943). Furthermore, Smith and Cook (1953) have demonstrated that 0.25 M KCl will separate a carrageenan solution into two components. The precipitate is designated kappa (κ-) carrageenan

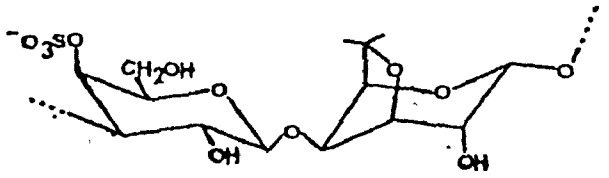
and is the gelling component. Lambda (λ-) carrageenan remains in solution. The proportion of κ-carrageenan varies in the different seaweeds and in a single species with respect to season and habitat (Black et al., 1965). Pernas et al. (1967) fractionated carrageenans from C. crispus, Gigartina stellata and G. skottsbergii at different KCl concentrations. Their evidence suggested there was a spectrum of molecules having different solubilities, hence they postulated that carrageenans are more heterogeneous than just the kappa and lambda components. Recent evidence (McCandless et al., 1973) showed that the gametophytic plant produced carrageenan of the kappa (κ-) family and the tetrasporic plant produced lambda (λ-) carrageenan. McCandless and Craigie (1974) have suggested that the variation reported earlier arose from the relative abundance of the different stages in the life cycle. The spectrum of polysaccharides observed by Pernas et al. (1967) may also be interpreted as due to this phenomenon.

Besides being of commercial value carrageenans are also important to the plant from which they are extracted. Gordon and McCandless (1973) have demonstrated that κ-carrageenan is concentrated in the immediate cell wall of all cells of both cortex and medulla of certain plants. Subsequently, κ-carrageenan was shown to be concentrated in the immediate cell wall of the gametophytic plant only and

λ- carrageenan was concentrated in the immediate cell walls of the tetrasporic plant. Rees (1962) has suggested that sulphated polysaccharides may function to (i) provide a cation exchange barrier between protoplasm and sea water, (ii) provide by their hydrophilic nature a reservoir of moisture which reduces the danger of desiccation during low tide (iii) provide a cushion to protect the cells from physical buffeting.

Chemistry of Carrageenans

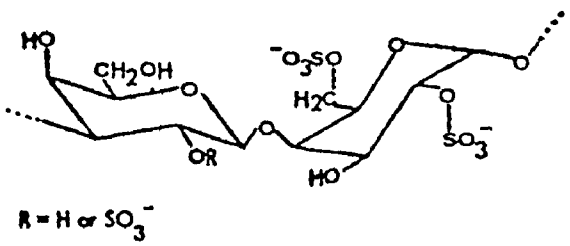
Carrageenans were originally shown to consist of three main components; 3,6-anhydro-D-galactopyranose, D-galactopyranose, and sulphate half ester groups (O'Neill, 1955). The KCl insoluble fraction had a high content of 3,6-anhydro-D-galactopyranose and the soluble fraction had only trace amounts (Rees, 1963; Dolan and Rees, 1965). It is now generally recognized that kappa carrageenans consist of repeating units of O-β-D-galactopyranosyl-4-sulphate (1 → 4)-O-α-3,6-anhydro-D-galactopyranose (1 → 3) (Dolan and Rees, 1965).



Repeating unit of kappa carrageenan

A galactan chemically similar to κ-carrageenan has been extracted from certain species of Eucheuma (Mueller and Rees, 1968). This has been designated iota (ι) carrageenan. It differs from κ-carrageenan by the presence of a 2-sulphate on some of the β (1,4) linked units.

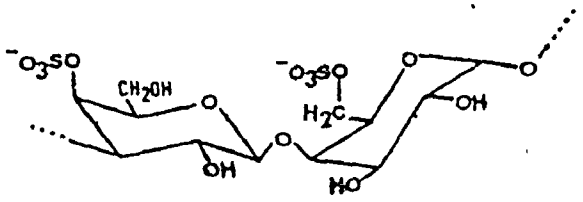
Lambda carrageenan by definition does not contain any galactopyranose 4-sulphate or 3,6-anhydro-galactopyranose (Dolan and Rees, 1965). It is thought that its structure consists of repeating units of O-β-D-galactopyranosyl or D-galactopyranosyl-2-sulphate (1 → 4)-O-α-D-galactopyranose-2,6-disulphate (1 → 3).



Repeating unit of lambda carrageenan

Another carrageenan type was identified by Dolan and Rees (1965). This was named mu (μ-) carrageenan. It has since been discovered that treatment of the KCl soluble fraction from C. crispus with alkaline borohydride yields a product which can be subfractionated with KCl (Anderson et al., 1968). The authors postulated that by alkaline borohydride treatment κ-carrageenan is formed from μ-carrageenan by the release of 6-sulphate from the α (1,3) linked unit

with concomitant formation of 3,6-anhydro-D-galactopyranose. It is tempting to postulate that in nature μ- carrageenan is the precursor of κ- carrageenan and that an enzyme present in the plant can perform the conversion. Such an enzyme has been discovered in G. stellata (Lawson and Rees, 1970).



Repeating unit of mu carrageenan

Immunochemistry of Carrageenans

Carrageenan was shown to be capable of stimulating connective tissue growth (Robertson and Schwartz, 1953). McCandless (1967) found that connective tissue growth could be further exagerated by a second injection of carrageenan. This implied that there was some memory mechanism in the animal and possibly that the carrageenan possessed immunologic activity. Johnston and McCandless (1968) later discovered that injection of λ- or κ- carrageenan from C. crispus into the marginal ear vein of rabbits resulted in the formation of precipitating

antibodies in the serum. These antibodies were shown to be specific; anti λ- carrageenan failed to precipitate κ- carrageenan and anti κ- carrageenan failed to precipitate λ- carrageenan. It was also verified that precipitin curves obtained by plotting the amount of antibody precipitated by increasing amounts of antigen conformed to the equations originally proposed by Heidelberger and Kendall (1935a,b) to describe the reaction. Subsequent work (Johnston and McCandless, 1969) has shown that anti λ- carrageenan (C. crispus) will precipitate λ- carrageenan fractions (soluble in 1.5 M KCl) from G. acicularis, G. pistillata, G. radula. Kappa carrageenan fractions (insoluble in .0625 M KCl) from the different species failed to precipitate anti λ- carrageenan. The converse experiment using anti κ- carrageenan (C. crispus) was also true. Anti κ- carrageenan precipitated the κ- carrageenan fractions from the various species but did not precipitate the λ- carrageenan fractions. In addition it was shown that carrageenans precipitated by intermediate KCl concentrations (0.06 - 1.5 M) from the different species could not be equated to each other immunochemically.

MATERIALS AND METHODS

Carrageenans

Carrageenans were extracted and fractionated from the tetrasporic and the carposporic stages of the alga according to the technique of McCandless et al. (1973). The lambda (λ-) carrageenan used was the 0.3 M KCl soluble fraction of the tetrasporic plant and kappa (κ-) carrageenan was the 0.3 M KCl insoluble fraction of the carposporic plant. For immunologic studies these were dissolved in 0.01 M phosphate buffered saline (PBS), pH 7.4 (0.9% NaCl). Carrageenans were analyzed for 3,6-anhydrogalactose by the modified resorcinol test of Yaphe and Arsenault (1965). Sulphate analyses were performed according to the technique of Jones and Letham (1954).

Algal Species

Dried specimens of tetrasporic and gametophytic stages were obtained from a number of sources. Information on these is summarized in Table I.

Immunization

Goats were injected with carrageenan derived from C. crispus, conjugated to methylated bovine serum albumin (mBSA). Methylated BSA was prepared by adding one gram

Table I

Source of Carrageenans

Algal species	Stage of life cycle	Source
Chondrus crispus	tetrasporic	Dr. Frank Loewus State University of New York at Buffalo, Buffalo, N.Y.
	carposporic	Atlantic Regional Laboratory, N.R.C.C. Halifax, Nova Scotia
Eucheuma spinosum (supplied as iota carrageenan)	"iota" Rees	W.A.P. Black Arthur D. Little Research Institute Musselburgh, Midlothian
	"iota" 6060	Marine Colloids Inc. P.O. Box 308 Rockland, Maine
Furcellaris fastigiata (supplied as furcellaran)	tetrasporic	Dr. Jorgan Christiansen Litex Ltd. Glostrup, Denmark
	undetermined stage (A949)	
Gigartina agardhii	carposporic	Dr. John West University of California Berkeley, California
Gigartina corymbifera	tetrasporic	Dr. Janet Stein University of British Columbia Vancouver, B. C.
Gigartina papillata	carposporic	Dr. Janet Stein University of British Columbia Vancouver, B. C.

TABLE I (cont'd)

Algal species	Stage of life cycle	Source
Gigartina papillata	carposporic	Dr. John West University of Californi Berkeley, California
Gigartina stellata	carposporic	Prof. J.Y. Floc'h Laboratoire de Physiologie Végétale Faculte des Science Brest, France
Hypnea musciformis	carposporic	Mr. Jean Mollion Dakar, Senegal
	tetrasporic	"
Iridaea cordata	carposporic	Mrs. Judy Hansen Division of Natural Science University of Californi Santa Cruz, California
	tetrasporic male	"
Petrocelis franciscana	tetrasporic	Dr. John West Department of Botany University of Californi Berkeley, California

of BSA to 100 ml absolute methanol. To this was added 0.84 ml concentrated KCl to aid in the dissolution of BSA. When BSA was dissolved the beaker was fully covered with aluminum foil and placed in a dark room at room temperature. The beaker was kept in the dark and was agitated once a day for 3 days, for 10 minutes. The precipitat was then spun down at 15,000 g in a Sorvall centrifuge. The pellet was washed twice in anhydrous ether. The washed precipitate was then stored in vacuo overnight at room temperature over KOH. A 3 mg/ml solution made in PBS, pH 7.4 was sterilized by passage through a 40 μ millipore filter. An equal amount of mBSA by weight was added to a sterile carrageenan solution. The resulting solution was allowed to stand overnight at room temperature, after which the precipitate was spun down at 3,000 rpm for 20 minutes. The supernatant was decanted and the precipitate redissolved in 10 ml sterile PBS.

Goats were injected subcutaneously in multiple sites in the shoulder region with the conjugated carrageenan solution in complete Freund's adjuvant (CFA). Blood samples were taken from the external jugular vein of the goat prior to injection and at weekly intervals post injection. Serum was obtained by incubating the blood at 37°C for 3 hours and then overnight at 4°C. The clot was removed and the serum was clarified by

spinning at 10,000 g for 30 minutes. When the serum showed specific activity with the homologous antigen the animal was anesthetized and exsanguinated by cannulating the carotid artery. Sera were sterilized by passage through a 40 μ millipore filter into previously sterilized serum hypovials. Vials were stored at -70°F.

Titres of Antibody

Immune sera were assayed for antibody titre according to the procedure of Martin (1943). This involved first setting up an antigen dilution series with a constant amount of antiserum to find the smallest amount of antigen which will precipitate a constant amount of antibody. If the amount of antigen used were greater than this the endpoint of precipitation would lie in the inhibition zone (the region of antigen excess) and the antibody titre would depend upon the amount of antigen used in the test as well as the potency of the antiserum. A second series of tubes was then set up using this amount of antigen and progressively decreasing amounts of antiserum. The titre obtained is the reciprocal of the least amount of antibody which still shows visible precipitation. Serum dilutions of 1:25 to 1:12,800 were used in conjunction with antigen concentrations of 1.0 μg/ml to 500 μg/ml. A total volume of 1 ml was maintained, of which the serum sample was 0.5 ml. Tubes were inspected daily for the presence of

precipitation.

Qualitative Precipitin Reaction

The qualitative precipitation reaction was performed in 0.75% agarose gels. One central well and six peripheral wells were cut in the gel. Antiserum was placed in the central well and the appropriate antigens were placed in the peripheral wells. The plates were allowed to develop 15 hours after which time precipitin lines were observed. Photographs were taken in order to secure a permanent record. The plates were flooded with distilled water and contact prints were taken directly on #5 photographic paper. Photographs of the prints were taken with a Pentax camera. The negative was printed on high contrast paper.

Quantitative Precipitin Reaction

The precipitin reaction was quantitated by the technique of Heidelberger and Kendall (1929, 1933, 1935). A preliminary antigen dilution series was set up to find the correct range of antigen concentration. Increasing amounts of antigen were added to 1 ml of antiserum diluted 1:5. A total volume of 2 ml was maintained by adding the appropriate amount of PBS. The tubes were incubated at 37°C for 2 hours and then 5 days at 4°C. Precipitates were harvested by centrifuging at 6,700 g for 30 minutes.

The precipitate was then washed two times with 0.01 M PBS, pH 7.4. After the final washing the precipitate was analyzed for protein by the Folin Ciocalteau method of Lowry et al. (1951) using BSA as standard.

Characterization of the Antibody

Isolation of gamma globulin by ammonium sulphate precipitation.

Gamma globulin was precipitated from immune serum with 33% $(NH_4)_2SO_4$ as follows: serum was clarified by centrifuging at 10,000 g for 30 minutes and an equal volume of 0.9% NaCl was then added; 20 ml saturated ammonium sulphate (SAS) was added dropwise to the serum solution, while it was continuously stirred. Following the addition of SAS, the pH was adjusted to 6.5 with 0.5 M NaOH and the solution was stirred for an additional 30 minutes. The precipitate was harvested by centrifuging at 20,000 g for 20 minutes. The precipitate was washed twice with 40% $(NH_4)_2SO_4$. After the final washing the precipitate was redissolved in 40 ml of 0.9% NaCl and the gamma globulin was reprecipitated with SAS, and washed twice with 40% $(NH_4)_2SO_4$. The washed precipitate was dissolved in 10 ml 0.01 M PBS, pH 7.4, and dialysed for one week against neutral 0.9% NaCl. The NaCl solution was changed daily during the week of dialysis.

Electrophoresis

The following goat sera were separated by electrophoresis on cellulose acetate strips:

immune serum

immune serum minus gamma globulin

gamma globulin

A small sample applied at the cathode using a Gelman applicator was electrophoresed in barbital buffer pH 8.6, ionic strength 0.1 for 45 minutes at 350 V. The strips were stained in Ponceau S for 5 minutes and destained by washing 3 times in 5% acetic acid. The strips were cleared by placing in 10% acetic acid in methanol for 60 seconds and dried by heating for 15 minutes at 60^{o}C. Cleared strips were scanned in a Beckman densitometer.

RESULTS

Characterization of the Antiserum

The antigenic nature of κ- and λ- carrageenans was first described in rabbits (Johnston and McCandless, 1969). They found that a detectable level of serum antibodies was present four days after an intravenous injection of 1 mg of λ- carrageenan. Initial attempts in the present work, therefore, were directed towards immunization of rabbits against κ- and λ- carrageenans. The animals were injected subcutaneously with 1 mg of antigen at weekly intervals for two months. However, precipitating antibodies were not detected during this period. Because of the failure to obtain antibodies in rabbits, in the present study goats were used since antibody production in this species had been successful in the work of Gordon and McCandless (1973).

Two goats were injected subcutaneously with carrageenan conjugated to mBSA mixed with Freund's complete adjuvant. Goat 7 was injected with conjugated λ- carrageenan and goat 9 was injected with conjugated κ- carrageenan. Immune serum from goat 9 showed specific activity; the serum was precipitated by C. crispus female insoluble (κ-) carrageenan but not by C. crispus tetrasporic soluble (λ-)

carrageenan (Figures 1A and 4A).

Immune sera from goat 9 was analyzed by electrophoresis on cellulose acetate strips. The result illustrated in Figure 2 shows that the serum contains a large amount of protein that bands in the gamma region. Isolated gamma globulin was tested to see if it possessed the same properties as whole serum. Figure 3 reveals that gamma globulin does in fact precipitate κ- carrageenan and it does not precipitate λ- carrageenan. Furthermore, serum from which gamma globulin had been removed failed to precipitate the carrageenans. Therefore the serum reactive substance appears to be an antibody of either the IgG or IgM class since these are the two known precipitating antibody classes which migrate in the gamma globulin region.

Goat 7 serum also developed a high titre of precipitating antibodies but the reaction was not specific. Therefore in order to render the serum useful it was necessary to absorb out the contaminating antibodies by adding an appropriate amount of κ- carrageenan. In this instance 1 ml of serum was absorbed with 275 µg of κ- carrageenan. The absorption was allowed to take place for 3 days. As expected, this serum was specific; it precipitated C. crispus tetrasporic soluble (λ-) carrageenan but not C. crispus female insoluble (κ-) carrageenan (Figure 1B. See also Figure 13A.)

The antibody titre of goat 9 (anti kappa carrageenan serum) was analyzed by the method of Martin (1943). The results are tabulated in Table II. It is evident that the endpoint titre of the antiserum varies with the amount of antigen added. The titre 2560 is the reciprocal of the maximum serum dilution which still precipitated the antigen.

Immunochemical Studies

Goat 9 anti κ- carrageenan was shown by immunodiffusion test to be precipitated not only by the homologous antigen C. crispus female insoluble carrageenan, but also by female insoluble carrageenans from Gigartina papillata, G. agardhii, G. stellata and H. musciformis (Figure 4B). This result is verified by a quantitative precipitation test (Figure 5). However, this latter analysis further reveals that carrageenans from species other than C. crispus precipitated less protein and had unique precipitin curves, with a narrower equivalence zone especially in the case of G. papillata carrageenan. The antiserum also precipitated female soluble carrageenans from C. crispus, G. agardhii and G. papillata (Figure 6A); however, it was noted that the precipitin lines on the Ouchterlony plates were less intense than with female insoluble carrageenan. Figure 7 illustrates that the female soluble carrageenans precipitate substantially less protein than their female insoluble

Table II

Martin Square Test for Antibody Titre

Antiserum, Anti kappa Carrageenan, G-9

Serum dilution	Antigen, female insoluble carrageenan, C. crispus µg/ml									
	512	256	128	64	32	16	8	4	2	1
1:5	+	+	+	+	+	+	+	+	+	+
1:10	+	+	+	+	+	+	+	+	+	+
1:20	+	+	+	+	+	+	+	+	+	+
1:40	+	+	+	+	+	+	+	+	+	+
1:80	+	+	+	+	+	+	+	+	+	+
1:160	+	+	+	+	+	+	+	+	+	+
1:320	-	+	+	+	+	+	+	+	+	+
1:640	-	+	+	+	+	+	+	+	-	-
1:1280	-	-	-	+	+	+	-	-	-	-
1:2560	-	-	-	-	-	+	-	-	-	-
1:5120	-	-	-	-	-	-	-	-	-	-
1:10240	-	-	-	-	-	-	-	-	-	-

Serum volume: 0.5 ml

Total volume: 1.0 ml

(+) precipitation

(-) no precipitation

counterparts. Tetrasporic soluble carrageenans from C. crispus, P. franciscana and G. corymbifera did not precipitate anti kappa carrageenan serum (Figure 6B).

After treatment of C. crispus female soluble carrageenan with alkaline borohydride the resulting insoluble fraction, μ converted to κ- carrageenan (μ → κ), precipitated slightly more protein than the homologous antigen (Figure 8). The remaining soluble fraction (x) precipitated a comparatively small amount of protein, and no line could be seen on the Ouchterlony plate (Figure 9B). Alkaline borohydride treated λ- carrageenan also precipitated the anti serum (Figure 9A). A faint precipitin line can be distinguished between the centre well and wells 3 and 6.

Carrageenans from another species of algae, Iridaea cordata, were also tested immunochemically to determine whether different stages of the life cycle produce antigenically distinct carrageenans in this genus as well as in Gigartina and Chondrus (Figure 10). Both male and female soluble and insoluble carrageenans from I. cordata precipitated the anti κ- carrageenan serum. The tetrasporic soluble fraction did not react.

Hypnea musciformis, however, proved to be different from the other algae since female insoluble and tetrasporic soluble carrageenans both reacted with the anti κ- carrageenan serum (Figure 11B).

Other polysaccharides which possessed "kappa-like" properties included iota carrageenan (Figure 11A) and furcellaran (Figure 12). The two iota carrageenan preparations tested reacted with the anti κ- carrageenan serum but differences between them could be seen in the Ouchterlony plates. Iota Rees precipitated the anti serum much closer to the antigen well than Iota 6060. In Figure 12 it can also be seen that furcellaran from tetrasporic plants precipitated the anti serum, as did furcellaran from a mixed population. Therefore, like H. musciformis its source Furcellaria fastigiata represents another species which is different from those tested in the genera Chondrus, Gigartina and Iridaea which produce different types of carrageenan in the different stages.

The lambda - anti lambda carrageenan system seems to be somewhat more complex than the previous system. Absorbed anti lambda carrageenan was specific since it did not react with kappa carrageenan (Figure 13A). But the reactions with tetrasporic soluble and female soluble carrageenans of the various species yielded one, two or three precipitin lines (Figure 13B). Tetrasporic soluble carrageenans from P. franciscana and G. corymbifera reacted with the anti lambda carrageenan serum but they precipitated less protein than the homologous antigen (Figure 14).

The female soluble fraction from C. crispus precipitated as much protein as the tetrasporic soluble fraction from the same algae (Figure 15). Female soluble fractions from G. agardhii and G. papillata also precipitated an appreciable amount of protein (Figure 16).

Figure 1

A Precipitation of goat 9 anti kappa serum by

- ☒ C. crispus female insoluble carrageenan
- ☐ C. crispus tetrasporic soluble carrageenan.

B Precipitation of absorbed goat 7 anti lambda serum by

- ☐ C. crispus tetrasporic soluble carrageenan
- ☐ C. crispus female insoluble carrageenan.

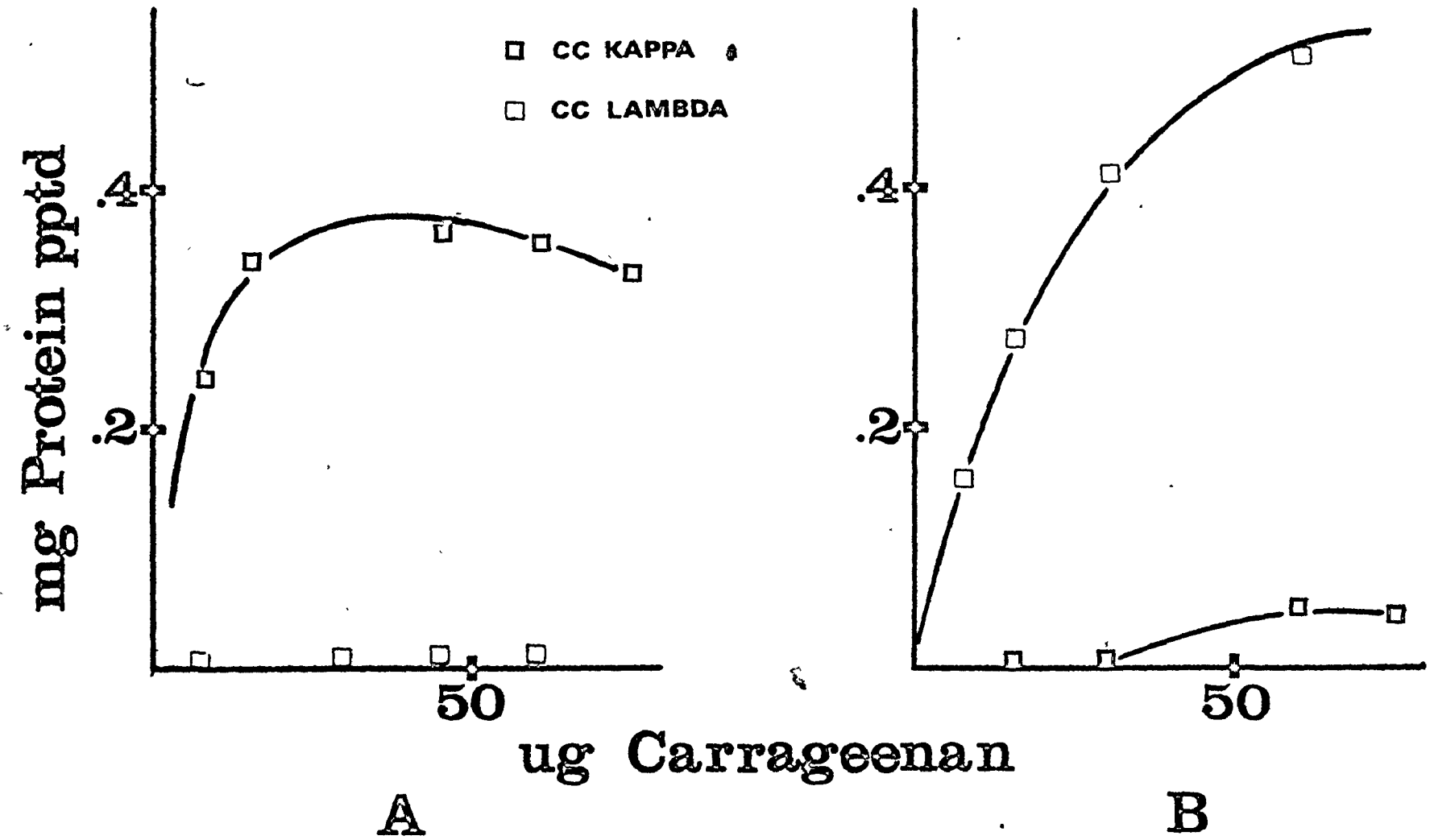

CC KAPPA
CC LAMBDA
mg Protein ppt'd
.4
.2
50
ug Carrageenan
A
B

Figure 2

Electrophoretic patterns of

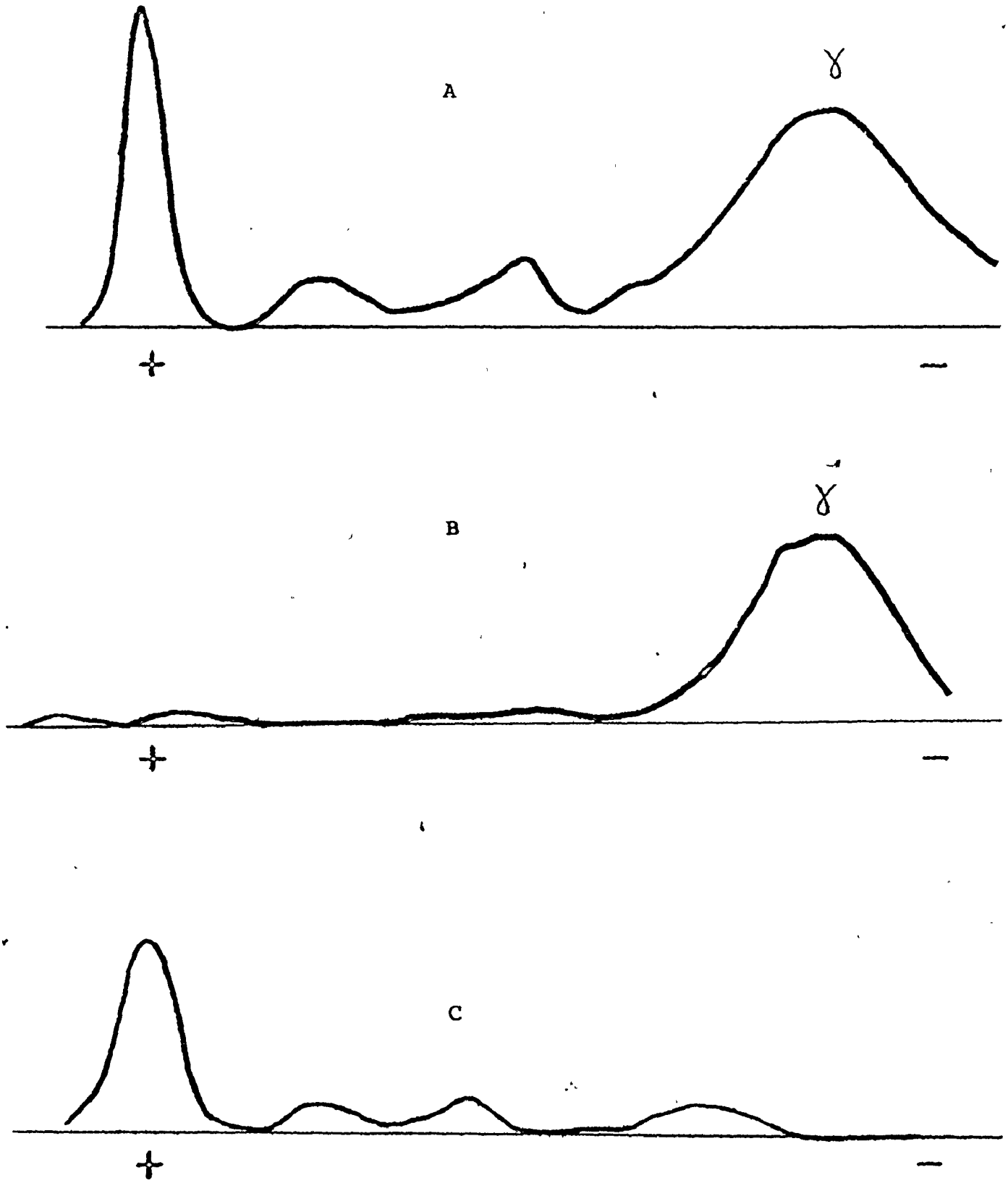
A goat 9 immune serum

B goat 9 gamma globulin (33% ammonium sulphate precipitate)

C goat 9 immune serum minus gamma globulin (33% ammonium sulphate supernatant)

Buffer, barbital pH 8.6

Stained with Ponceau S


A
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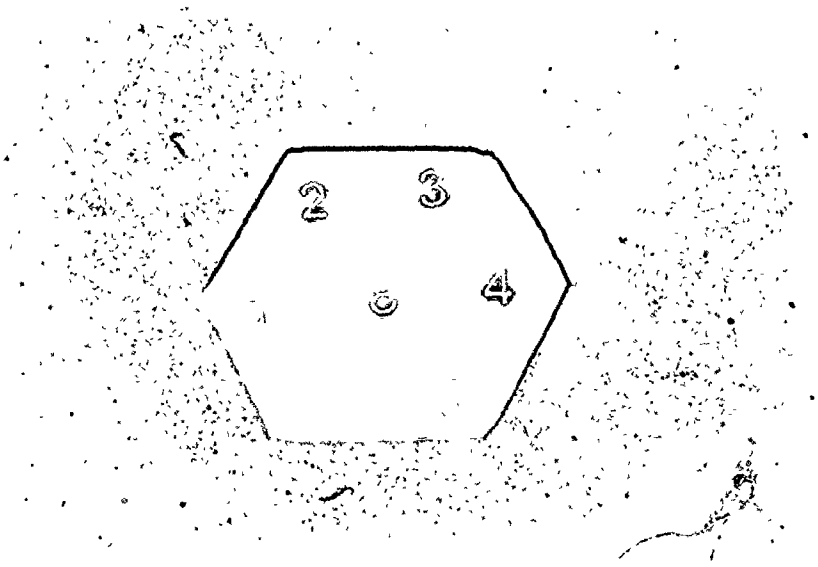


Figure 3

Centre well - goat 9 gamma globulin

Peripheral wells 1) and 4) C. crispus female insoluble carrageenan 3) and 6) tetrasporic soluble carrageenan 2) and 5) goat 9 serum without gamma globulin.

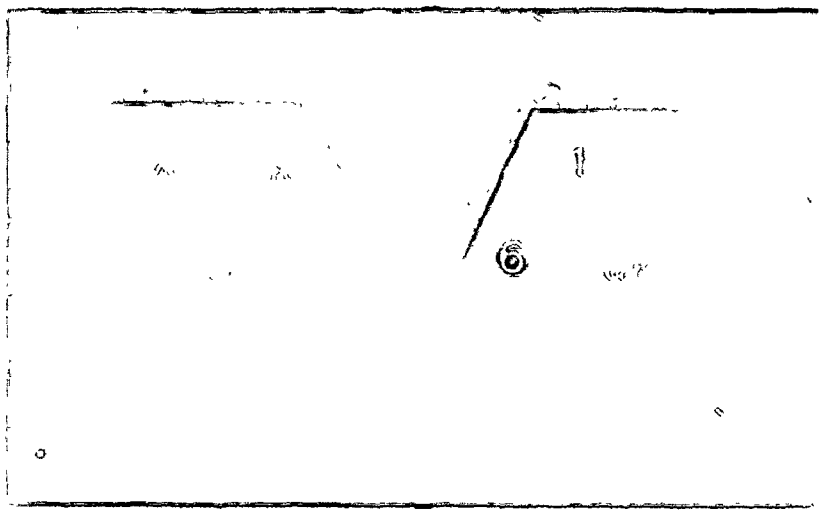


Figure 4

A: Centre well - goat 9 anti kappa serum.
Peripheral wells - λ, C. crispus tetrasporic soluble carrageenan. κ, C. crispus female insoluble carrageenan.

B: Centre well - goat 9 anti kappa serum.
Peripheral wells - female insoluble carrageenans extracted from 1) and 4) C. crispus 2) G. papillata 3) G. agardhii 5) G. stellata 6) H. musciformis.

Figure 5

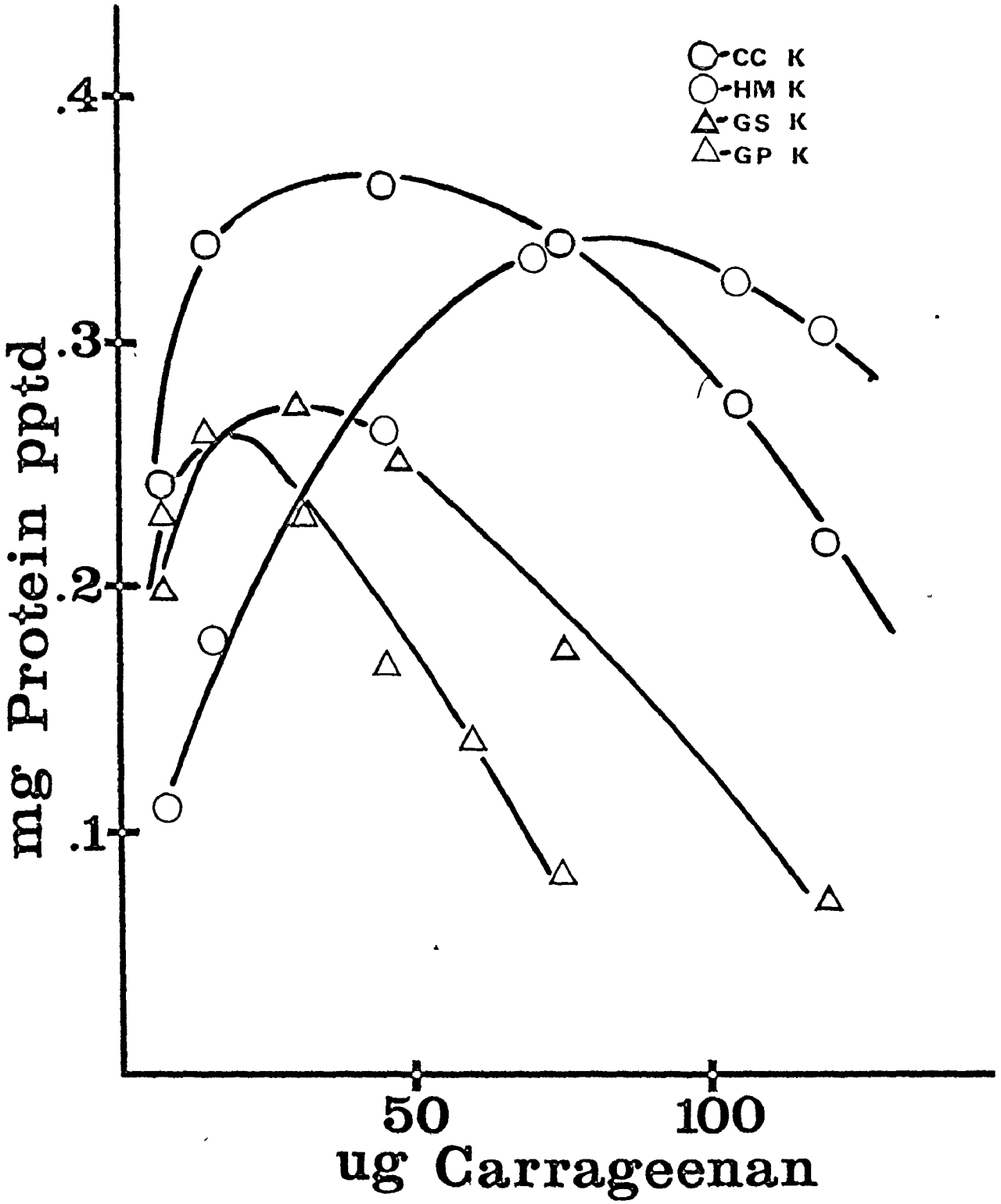
Precipitation of anti kappa serum by female insoluble carrageenans extracted from:

○ C. crispus

○ H. musciformis

△ G. stellata

△ G. papillata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HM K
GS K
GP K
.4
.3
.2
.1
mg Protein ppt'd
50
100
ug Carrageenan

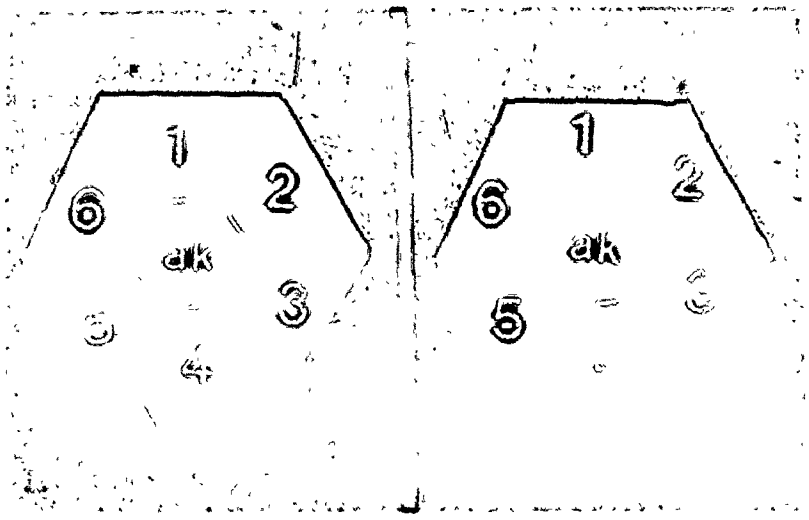


Figure 6

A: Centre well - goat 9 anti kappa serum.

Peripheral wells - 1) and 5) C. crispus female insoluble 2) G. agardhii female insoluble 3) and 6) G. agardhii female soluble 4) C. crispus female soluble.

B: Centre well - goat 9 anti kappa serum

Peripheral wells - 1) C. crispus tetrasporic soluble 2) P. franciscana tetrasporic soluble 3) G. corymbifera tetrasporic soluble 4) C. crispus female soluble 5) G. agardhii female soluble 6) G. papillata female soluble.

Figure 7

Precipitation of anti kappa serum by:

△ C. crispus female soluble

○ G. agardhii female soluble

△ G. papillata female soluble

○ C. crispus female insoluble

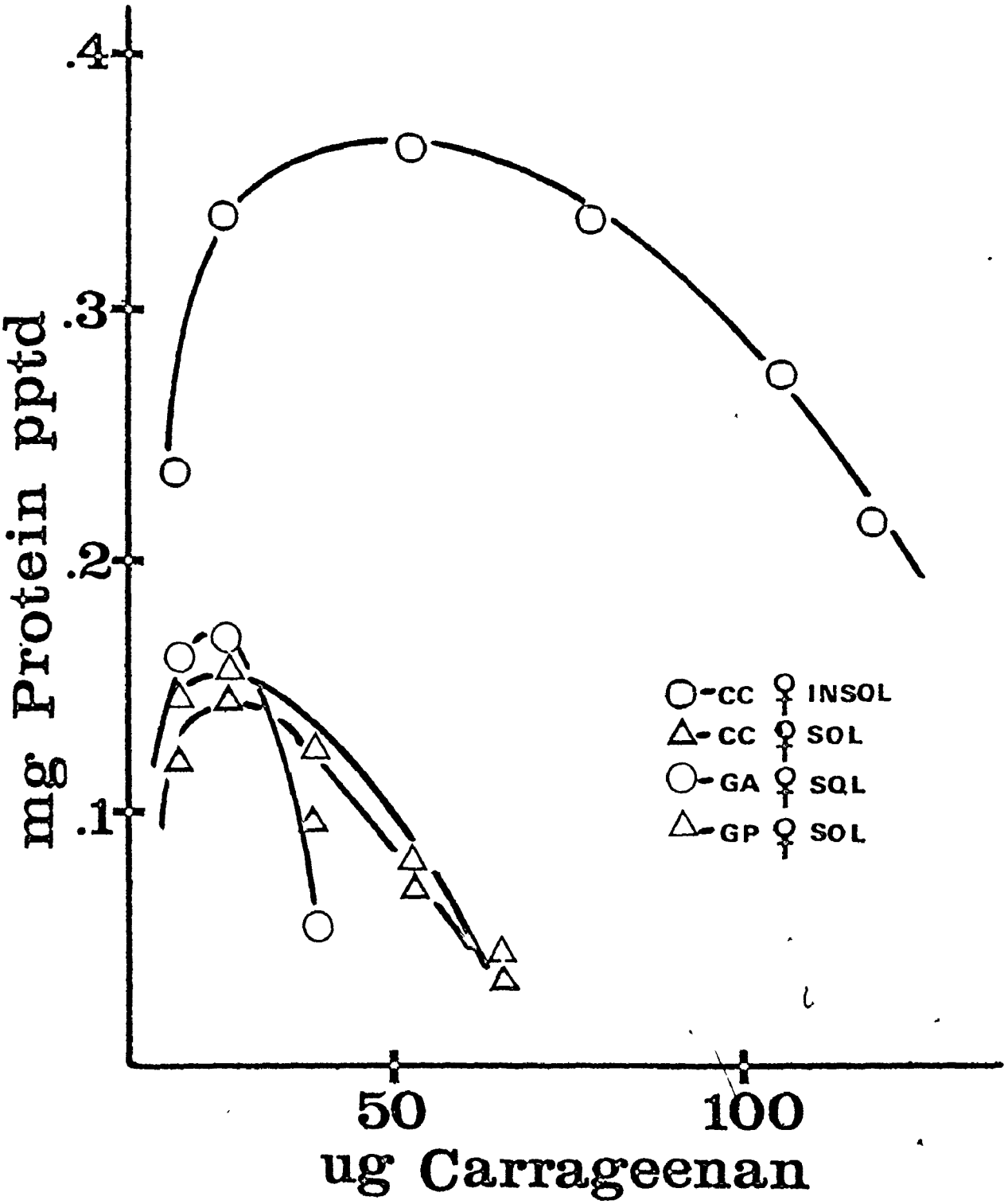

.4
.3
.2
.1
mg Protein pptd
50
100
ug Carrageenan
CC ♀ INSOL
CC ♀ SOL
GA ♀ SOL
GP ♀ SOL

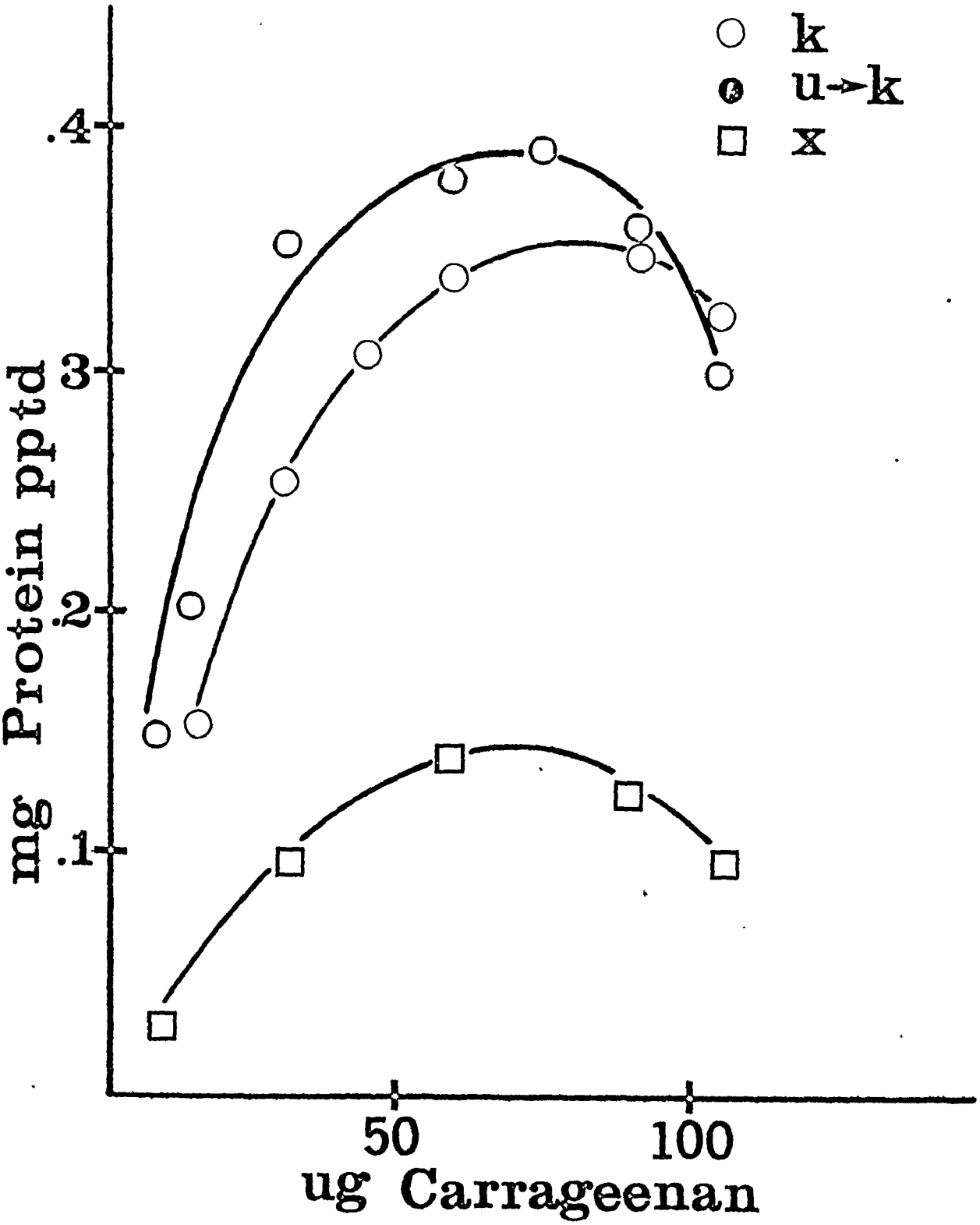
Figure 8

Precipitation of anti kappa serum by

○ C. crispus female insoluble

○ alkaline borohydride treated female soluble - precipitate (μ → κ)

□ alkaline borohydride treated female soluble - soluble fraction (x)


k
u→k
x
.4
.3
.2
.1
mg Protein ppt'd
50
100
ug Carrageenan

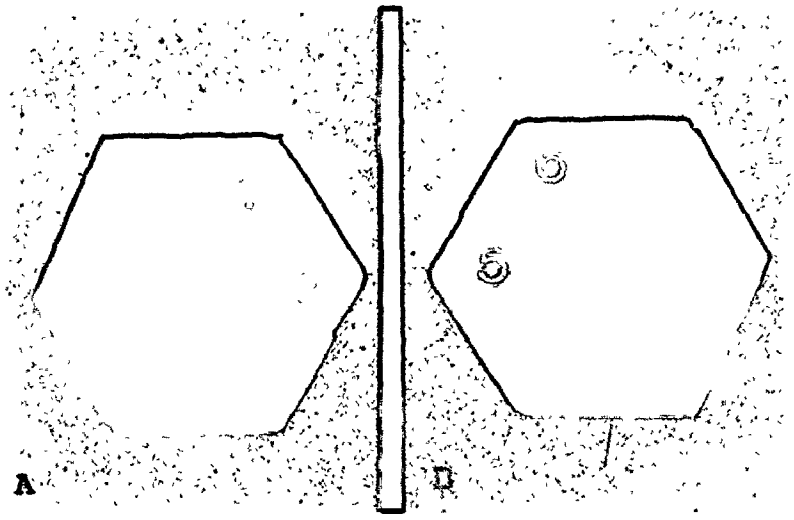


Figure 9

A: Centre well - goat 9 anti kappa serum.

Peripheral wells - 1) and 4) alkaline borohydride treated λ carrageenan from C. crispus (5055)

2) C. crispus female insoluble 3) and 6) alkaline borohydride treated lambda from C. crispus (15.3)

5) C. crispus tetrasporic soluble.

B: Centre well - goat 9 anti kappa serum.

Peripheral wells - C. crispus carrageenans 1) κ

2) and 5) μ + κ 3) and 6) x 4) λ.

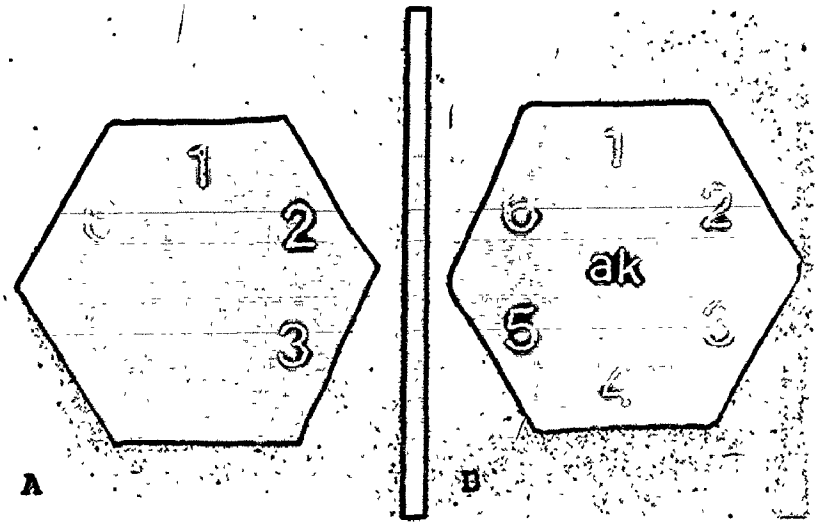


Figure 10

A: Centre well - goat 9 anti kappa serum.

Peripheral wells - 1) C. crispus female insoluble 2) and 5) I. cordata female insoluble 3) and 6) I. cordata female soluble 4) I. cordata tetrasporic soluble.

B: Centre well - goat 9 anti kappa serum.

Peripheral wells - 1) C. crispus female insoluble 2) and 5) I. cordata male insoluble 3) and 6) I. cordata male soluble 4) I. cordata tetrasporic soluble.

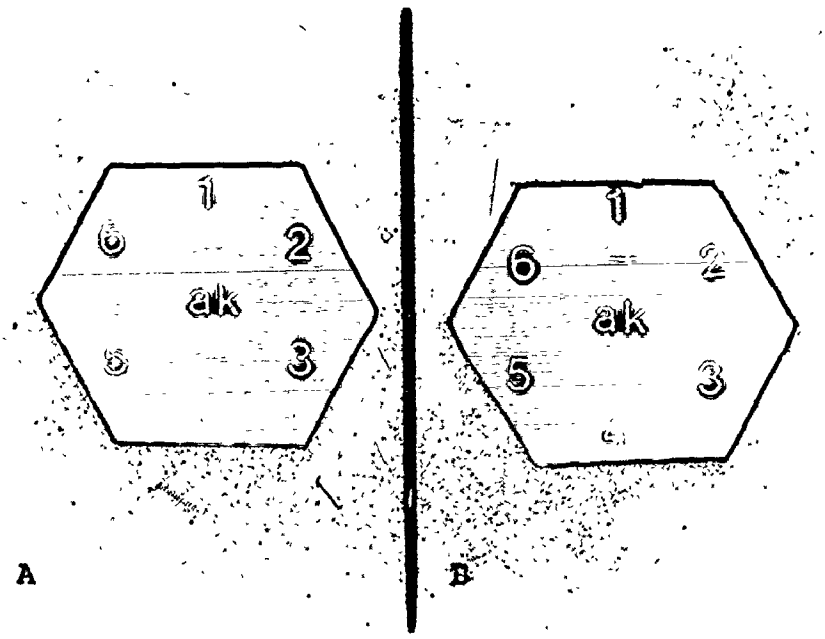


Figure 11

A: Centre well - goat 9 anti kappa serum.
Peripheral wells - 1) C. crispus female insoluble carrageenan 2) and 5) iota carrageenan (6060) 3) and 6) iota carrageenan (Rees) 4) C. crispus tetrasporic soluble.

B: Centre well - goat 9 anti kappa serum.
Peripheral wells - 1) C. crispus female insoluble 2) and 5) H. musciformis tetrasporic soluble 3) and 6) H. musciformis female insoluble 4) C. crispus tetrasporic soluble.

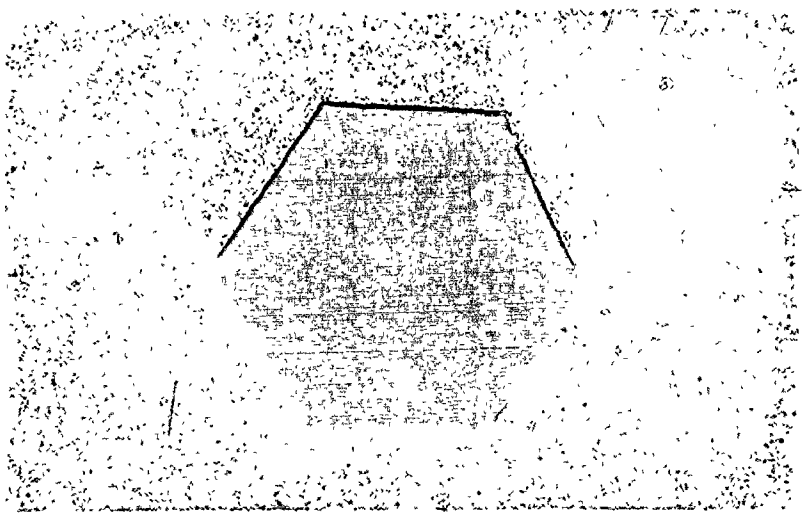


Figure 12

Centre well - goat 9 anti kappa serum.

Peripheral wells - 1) C. crispus female insoluble 2) and 5) furcellarum tetrasporic 3) and 6) furcellarum A949 4) C. crispus tetrasporic soluble.

Figure 13

A: Centre well - absorbed goat 7 anti lambda serum.
Peripheral wells - κ, C. crispus female insoluble.
λ, C. crispus tetrasporic soluble.

B: Centre well - absorbed goat 7 anti lambda serum.
Peripheral wells - 1) C. crispus tetrasporic soluble
2) P. franciscana tetrasporic soluble 3) G. corymbifera tetrasporic soluble 4) C. crispus female soluble
5) G. agardhii female soluble 6) G. papillata female soluble.

Figure 14

Precipitation of absorbed goat 7 anti lambda serum by tetrasporic soluble carrageenans extracted from

△ C. crispus

◇ P. franciscana

◇ G. corymbifera

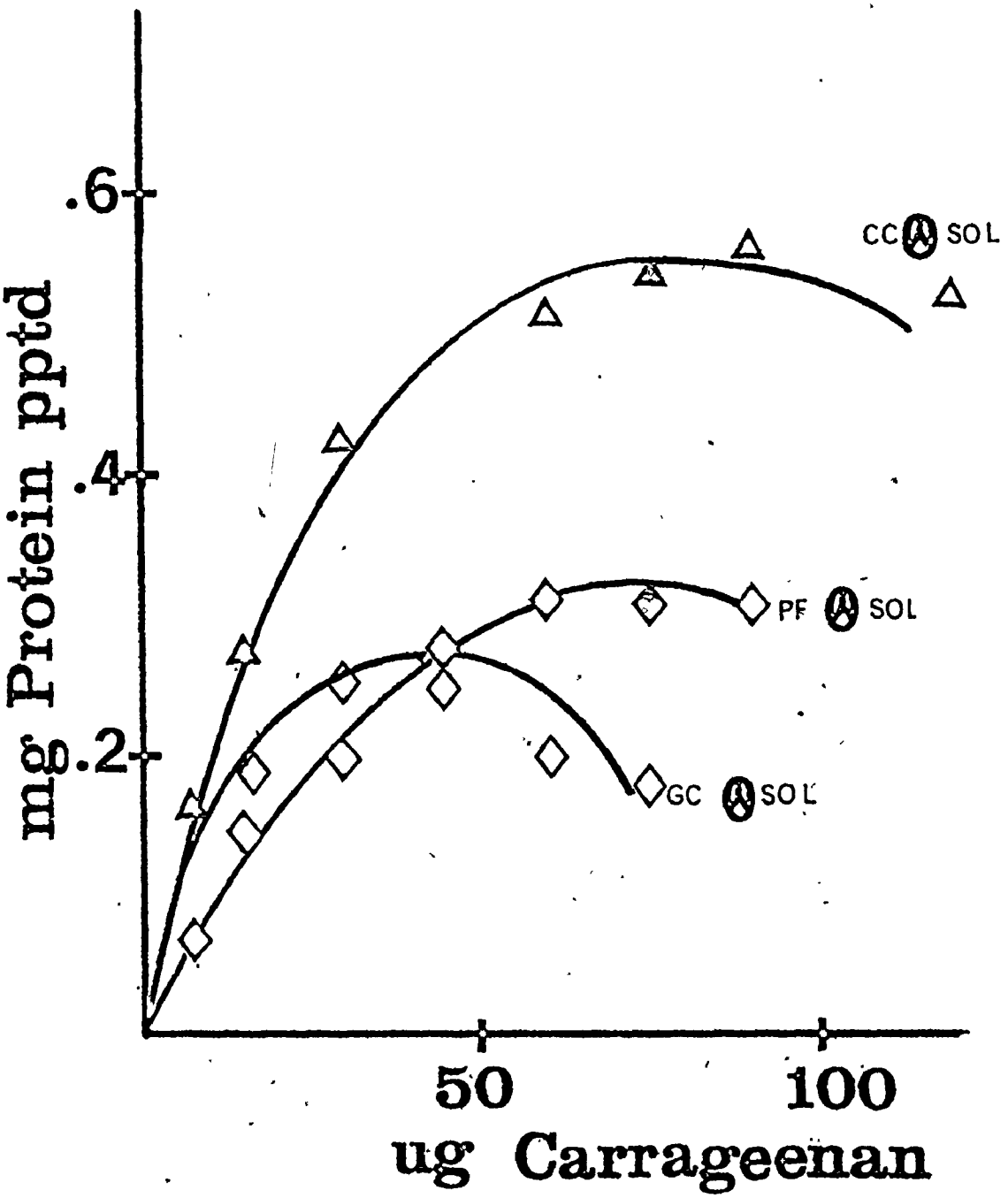

.6
.4
.2
mg Protein ppt'd
CC SOL
PF SOL
GC SOL
50
100
ug Carrageenan

Figure 15

Precipitation of absorbed goat 7 anti lambda serum by

△ C. crispus tetrasporic soluble

○ C. crispus female soluble

○ C. crispus female insoluble

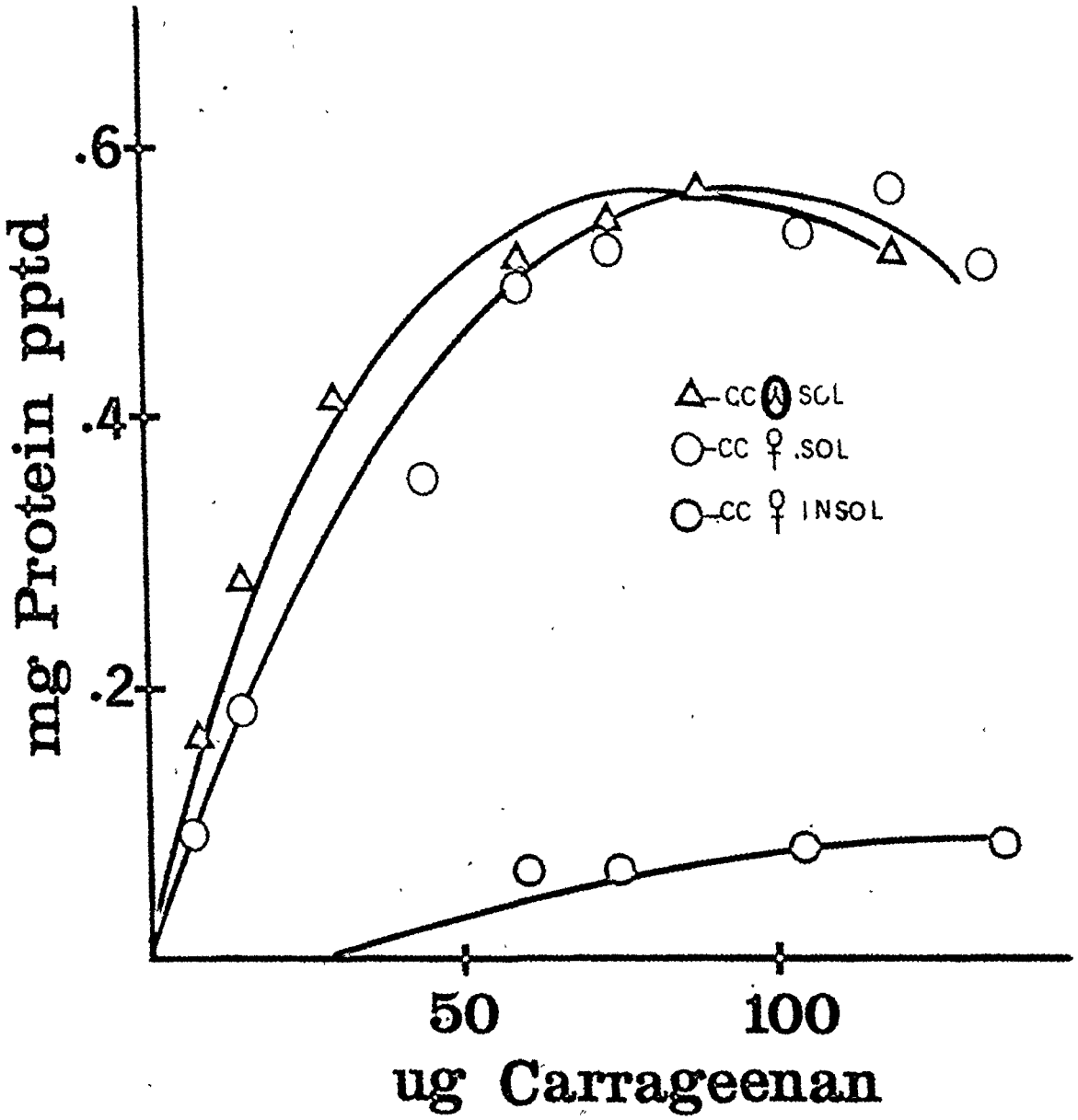

mg Protein pptd
.6
.4
.2
50
100
ug Carrageenan
CC ♂ SOL
CC ♀ SOL
CC ♀ INSOL

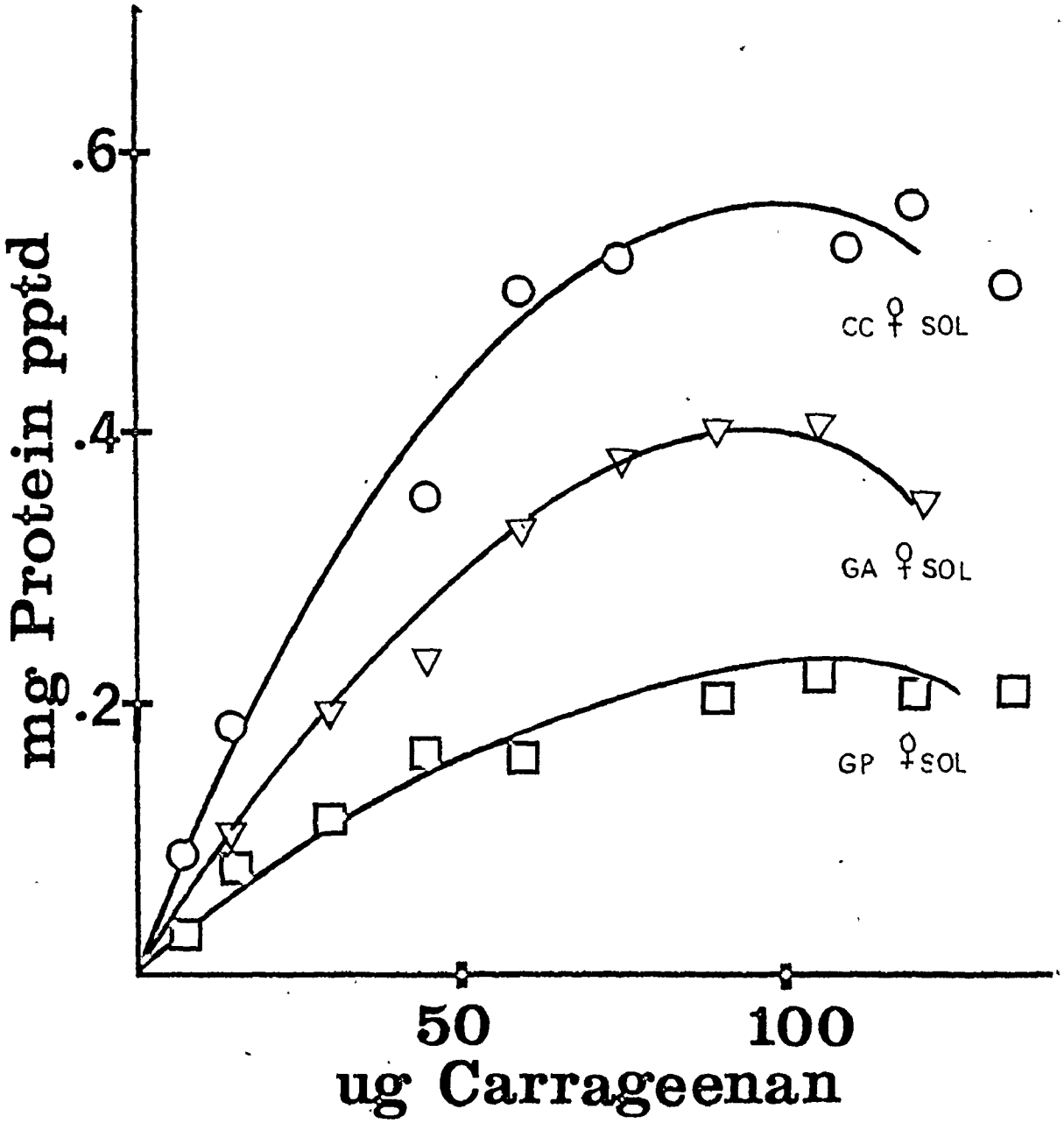
Figure 16

Precipitation of absorbed goat 7 anti lambda serum by:

○ C. crispus female soluble

▽ G. agardhii female soluble

□ G. papillata female soluble


.6
.4
.2
mg Protein pptd
CC ♀ SOL
GA ♀ SOL
GP ♀ SOL
50
100
ug Carrageenan

DISCUSSION

The Precipitin Reaction

The discovery and naming of the precipitin reaction was first attributed to Kraus (1897) who discovered that cell free filtrates of typhoid bacillus broth cultures precipitated anti typhoid serum. It is generally recognized to be a reaction between antigen and antibody which at the appropriate concentration of reactants (equivalence) results in the formation of an insoluble antigen-antibody complex. This idea is supported by electron micrographs of ferritin-antiferritin precipitates (Easty and Mercer, 1957). Since an antibody has at least two combining sites and an antigen has multiple determinant sites it is thought that the precipitate is formed as a lattice structure. The lattice is dependent upon a certain ratio of antigen to antibody. If the relative amount of antigen or antibody is too great the reaction is in antigen or antibody excess respectively and soluble complexes will remain in solution. In the quantitative precipitin reaction which has been perfected by Heidelberger and Kendall (1933 and 1935) a constant amount of antiserum was added to increasing amounts of antigen. The amount of antibody precipitated can be determined by analyzing

the washed precipitate. If the antigen is a carbohydrate then the amount of antibody precipitated is the total amount of protein present in the precipitate. Total protein can be analyzed by the Folin-Ciocalteau technique or protein nitrogen may be analyzed by the micro-Kjeldahl method. Since these procedures are extremely sensitive very small quantities of reagent may be used. Furthermore, the great sensitivity combined with specificity of the precipitin reaction allows for the detection of minute amounts of antigen. For example, Heidelberger and Kendall (1935) were able to detect 0.1 μg of egg albumin in 1.0 ml of fluid.

Another feature of the precipitin reaction is that it can be observed in gels. There are various ways of performing gel diffusion including simple diffusion in one direction, simple diffusion in two directions and double diffusion in one dimension, but the most useful method is double diffusion in two dimensions. When double diffusion is performed in two dimensions an agar or agarose layer is generally poured in a petri dish, wells are cut, and the gel removed from the well by suction. The central well may be filled with antibody and the various antigens may be placed in the peripheral wells. Although not so sensitive this method gives the same kind of information as the quantitative precipitin

tests. An added feature of gel diffusion techniques is that they can provide information as to the homogeneity and purity of antisera and antigens which is not obvious in the quantitative technique. Thus if the antigen is impure then the resulting antiserum will contain antibodies of different specificity. On an immunodiffusion plate this will be detected by the appearance of multiple precipitin lines in the gel.

Immunochemistry of Carrageenans

Specific antibodies have been used in the present study to determine the "kappa-like" and lambda-like" properties of carrageenans extracted from different stages in the life cycle of various species of red algae. Prior to 1973 it was commonly believed that the production of "κ-" and "λ-" carrageenans by C. crispus was influenced by seasonal factors (Black et al., 1965; Fuller and Mathieson, 1972). McCandless et al. (1973), however, discovered that the tetrasporic stage of this alga produced λ- carrageenan and the gametophytic stage produced κ-carrageenan with small amounts of μ- carrageenan and an additional KCl soluble carrageenan. More recently, McCandless (1975) investigated plants from known stages of the life cycle of species of red algae including Gigartina papillata, G. corymbifera, G. agardhii, G. stellata, Petrocelis franciscana and Hypnea musciformis. Her

data derived from sulphate, 3,6-anhydrogalactose, and infrared spectral analysis indicated that except for H. musciformis all sporophytic plants produced "lambda-type" carrageenan and all gametophytic plants produced "kappa-type" carrageenans. Because of the extreme precision and sensitivity of the immunological method it was of interest to reinvestigate this phenomenon using immunochemical techniques. Carrageenans from tetrasporic plants of C. crispus, G. corymbifera and P. franciscana precipitated anti λ- carrageenan serum but not anti κ-carrageenan serum. Carrageenans from gametophytic plants of C. crispus, I. cordata, G. stellata, G. agardhii and G. papillata precipitated anti κ- carrageenan serum. In two species C. crispus and I. cordata from which both stages were available the tetrasporic stage produced "lambda-like" carrageenan and the gametophytic stage produced "kappa-like" carrageenan. Only one stage was available in each of the other species. Thus, immunochemistry confirms the biochemical results.

Within the subgenus Mastocarpus which includes G. papillata, G. stellata and G. agardhii it appears that a tetrasporophyte is missing. However, it is possible that the tetrasporophyte exists as a phase different in morphology from the gametophyte. West (1972) has germinated tetraspores of the crustose species P. franciscana in culture and found that in 7 months these produced

reproductively mature plants which had similar morphology to G. agardhii. It is possible that a crustose species might also be the tetrasporic stage of G. stellata and and G. papillata but much more field ecology and laboratory culture work must be undertaken to substantiate this (West, 1972). As stated previously carrageenans from P. franciscana are "lambda-like" since they precipitated anti λ- carrageenan serum but not anti κ- carrageenan serum. Therefore, if P. franciscana is the tetrasporic stage of G. agardhii and hence belongs to the family Gigartinaceae then this would be another species which conforms to the phenomena since P. franciscana produces "λ-" carrageenan and G. agardhii produces "κ-" carrageenan.

Among the species investigated which produce κ- and/or λ- carrageenan an exception encountered was H. musciformis. In this species carrageenans from gametophytic and tetrasporic stages both precipitated the anti κ- carrageenan serum. It is interesting to note that Hypnea is classified in a different family from the rest of the algae studied. It belongs to the family Hypneaceae whereas Gigartina, Chondrus and Iridea belong to the family Gigartinaceae (Kylin, 1956). Another exception encountered was seen in Furcellaran fastigiata the source of furcellaran. Furcellaran is a polysaccharide with many similarities to κ- carrageenan including the fact that it contains approximately 30% 3,6-anhydrogalactose

and that it gels in the presence of potassium chloride. It differs in that only two in five galactose residues are sulphated (Percival and McDowell, 1968). In the Ouchterlony diffusion plate anti κ- carrageenan serum was seen to precipitate furcellaran from tetrasporic plants. Therefore furcellaran from tetrasporic F. fastigiata has κ- carrageenan properties. Again, this is different from the rest of the species studied in which tetrasporic plants all produced "lambda-like" carrageenans. However, F. fastigiata belongs to Furcellariaceae, a separate family from Gigartinaceae and thus is like H. musciformis. There is also an indication that Eucheuma nudum which belongs to the family Soleriaceae makes similar (iota) carrageenans in both tetrasporic and gametophytic stages (McCandless, personal communication). Therefore, evidence from infrared spectral analysis (McCandless, 1973) and the immunochemical evidence presented here suggest that so far species of Gigartinaceae and only species from this family conform to the Chondrus pattern whereby different stages in the life history produce different carrageenans. This assumes P. franciscana is the sporophyte of G. agardhii as suggested by West (1972). If Petrocelis franciscana is not the tetrasporic stage of G. agardhii then this would be an exception since theoretically Petrocelis belongs to the family Cruoriaceae

(Kylin, 1956) and not Gigartinaceae. Two other genera within Gigartinaceae remain to be investigated; Rhodoglossum and Besa.

The biochemical basis of the immunochemical difference between "κ-" and "λ-" carrageenans can be inferred from the structure of the two types of molecules. Kappa carrageenan differs from λ- carrageenan in that κ- carrageenan contains within its structure 4-sulphate and 3,6-anhydro-D-galactose moieties whereas λ- carrageenan does not. Lambda carrageenan contains galactose 2,6-disulphate which is not present in κ- carrageenan. The most obvious structural difference between female insoluble and tetrasporic soluble and insoluble carrageenans is the presence of 20-25% 3,6-anhydro-D-galactose in the former and only trace amounts, if any, in the latter (McCandless et al., 1973).

Female soluble carrageenans have approximately one half as much 3,6-anhydro-D-galactose as female insoluble carrageenans. As stated previously, anti κ- carrageenan serum precipitated female soluble carrageenan but to a lesser extent than female insoluble carrageenan. It did not precipitate tetrasporic soluble carrageenan. These data suggest that the antiserum is recognizing the 3,6-anhydrogalactose moiety or some structural feature attributed to it. More significant is the fact that after

the female soluble fraction was treated with alkaline borohydride which increased the 3,6-anhydrogalactose content this carrageenan (μ converted to κ- carrageenan) exhibited an increase in the amount of protein it precipitated from the antiserum.

Moreover, two alkaline borohydride treated λ-carrageenan preparations, "15-3" with 8.7% 3,6-anhydrogalactose and "5055" with 12% both precipitated anti κ-carrageenan serum. Untreated λ- carrageenan did not react. Other molecules which precipitated anti κ-carrageenan serum included furcellaran and iota carrageenan from Eucheuma spinosum. Both contained 3,6-anhydrogalactose.

The story of the anti λ- carrageenan serum is much less satisfactory and obviously much more work needs to be spent on this problem. Although there is not much evidence to implicate any structural groupings of the λ- carrageenan molecule which might act as a determinant there is one clue. The absorbed anti λ- carrageenan serum could not distinguish between female soluble and tetrasporic carrageenans. It precipitated with either of them. This suggests that the 6-sulphate residue might be important since this is the only frequently occurring structural feature shared by the two molecules.

The significance of the phenomena whereby the different stages synthesize different carrageenan molecules remains to be determined. Why should the different

stages possess such different molecules in their cell walls? Why should this phenomena be restricted to the Gigartinaceae if this is the case? Possibly it is a gene dosage effect since the tetrasporic stage is thought to contain twice as much genetic material as the gametophytic stage (Hanic, 1973).

The extra genetic material present in the sporophytes might have the effect of inhibiting an enzyme responsible for anhydrogalactose formation and it might induce sulfotransferases which sulphate on C_2 of alternate galactose units. But this is only speculation since very little is known about the genetics of the algae or the enzymes responsible for synthesizing the carrageenans. Fuller and Mathieson (1972) found at a number of different locations on the Eastern coast of the United States that littoral plants produced more κ- carrageenan than sublittoral plants. Therefore, in light of what is now known it seems reasonable to assume there are more gametophytic plants in littoral than sublittoral zones. Hence, it may be that the two stages occupy different ecological niches which inflict differential selective pressures on the cell wall components.

Obviously, there are many questions which remain to be answered and much more work needs to be done in the area of genetics, enzymology and ecology of the algae before the significance of the phenomena is understood.

SUMMARY

Quantitative and qualitative precipitin tests were performed on carrageenans extracted from stages in the life cycle of various species of red algae. Species from three genera in the family Gigartinaceae were studied and it was determined that specific anti κ-carrageenan serum was precipitated by carrageenans extracted from gametophytic plants but not by carrageenans from tetrasporic plants. A few species outside of the family did not conform to the phenomena.

Female soluble (0.3 M KCl) carrageenan precipitated less antibody than female insoluble (0.3 M KCl) but if the female soluble fraction were first treated with alkaline borohydride then it precipitated as much antibody as the female insoluble fraction. Alkaline borohydride treated λ- carrageenan also precipitated the anti κ- carrageenan serum. Other polysaccharides that precipitated anti κ-carrageenan serum include iota carrageenan and furcellaran.

Absorbed anti λ- carrageenan serum was precipitated by carrageenans from tetrasporic plants and female soluble carrageenans but not by female insoluble carrageenans.

The above precipitin reactions were also used to give some indication of the κ- and λ- carrageenan determinants. Evidence presented indicated that 3,6-anhydrogalactose is involved in the κ- carrageenan determinant. The nature of the λ- carrageenan determinant is unclear.

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