ANTIGENICITY OF CARRAGEENANS

THE IMMUNOLOGICAL RESPONSE IN RABBITS TO CARRAGEENANS, SULPHATED GALACTANS EXTRACTED FROM MARINE ALGAE

By

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

May, 1968

MASTER OF SCIENCE (1968) McMASTER UNIVERSITY
(Biology) Hamilton, Ontario

TITLE: The immunological response in rabbits to carrageenans, sulphated galactans extracted from marine algae

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NUMBER OF PAGES: v, 100

SCOPE AND CONTENTS:

Carrageenan, a mixture of sulphated galactans extractable by hot water from certain marine algae, has been known for its activity in a reversible stimulation of connective tissue growth. One subcutaneous injection of this polysaccharide elicits a granuloma formation which regresses after a few weeks. A single injection after regression results in heightened response; this suggested an immunological reaction. Serial injections of either lambda or kappa carrageenan into rabbits resulted in the formation of precipitins. The present study gives both qualitative and quantitative studies of the reaction involved. Fractions of carrageenans from different species of marine algae were tested for their ability to precipitate either anti-lambda carrageenan antiserum or anti-kappa carrageenan antiserum. Deductions were made from the data as to the structure of the polysaccharides.

ACKNOWLEDGEMENTS

I wish to acknowledge sincere gratitude to Dr. E. L. McCandless for her supervision, encouragement and generous assistance throughout this study. The author is grateful to Dr. O. Smidsrød (Trondheim, Norway) and Dr. B. Cinader (Toronto) for their interest and fruitful discussions concerning the present investigations. I wish to thank Miss I. L. Davy and Mrs. J. Astington for their technical assistance.

The generous supply of carrageenans by Marine Colloids, Inc., Rockland, Maine and by Dr. A. Haug, Norwegian Institute for Seaweed Research, Tronheim, Norway, is acknowledged.

The financial assistance of an Ontario Graduate Fellowship is acknowledged.

TABLE OF CONTENTS

INTRODUCTION

A. General considerations of antigenicity

The antigenicity of a substance is the sum of several factors; no one factor can be designated as the prime determinant in antigenicity. Antigens are foreign to the host; the metabolizability of the antigen and the ability of the host to "process" the molecule is important. A case in point is the poor antigenicity of rabbit haemoglobin in rabbits (Heidelberger and Landsteiner, 1923) but its effective antigenicity in chickens (Goodman and Campbell, 1954) and in guinea pigs (Ovary, 1964). Antigens must have more than a certain minimum degree of complexity and a certain molecular size. Proteins of high molecular weight, i.e., greater than 40,000, are good antigens and it is generally believed that molecules below 10,000 are poor antigens. However, low molecular weight polypeptides such as glucagon (molecular weight, 3485, Unger et al., 1959) and synthetic polymers of 3000 to 5000 mw can function as effective antigens (Maurer, 1963; Sela et al., 1962). The smallest immunogenic molecule for the guinea pig is tri-dinitrophenyl bactracin, mw 1928 (Maurer, 1965). With straight chained dextrans of mostly 1,6 alpha linkages, a molecular weight of 50,000 was highly antigenic; whereas when the degree of branching was increased, the molecule became more globular and a lower molecular weight polymer, 28,000, could elicit an antibody response (Kabat, 1961). Such factors will not only determine the antigenicity of the molecule with respect to its ability to

initiate antibody production but also its ability to form insoluble antigen-antibody complexes.

The importance of particle size in antigenicity is indicated by observations that non-antigenic or weakly antigenic substances can be made antigenic by adsorbing the molecule on a carrier such as kaolin, colloidin or charcoal. This presents more antigenic sites for reactivity. Haurowitz (1952) proposed the necessity of a rigid structure of the determinant groups. He suggested that the highly specific action of aromatic diazo compounds is due to the rigidity of their benzene rings; while the inability of long chain fatty acids to elicit antibody formation is due to the fact that paraffin chains are easily distorted and change their shape constantly. Sela and Fuchs (1963) have proposed that as little as a 2% increase in tyrosine content in the gelatin molecule would result in that molecule's becoming antigenic. Such assumptions are based on the premise that an antigen must have a definite configuration for it to be copied by the antibody forming mechanism and that this configuration must not be hidden in the interior of the molecule. Landsteiner (1945) proposed that charge has some importance; but this has been shown not to be essential. The neutrally charged blood group substances are highly antigenic and Sela and Fuchs (1963) have produced synthetic polypeptides devoid of charge that are antigenic. Thus, specificity of antigens resides in the structural peculiarities of their molecules. While it has not been possible in each case to correlate immunological differences in substances with their chemical structure, there is no doubt that a high degree of association does exist.

B. Antibody

The antibody molecule is a protein which has the property of being able to combine very selectively with the antigen against which it is directed. Antibodies found in the circulation are globulins (immunoglobulins) with a wide range of electrophoretic mobilities. They fall into several classes: IgG, IgM, IgA, IgD, IgE, IgT (equine), based on their electrophoretic and antigenic properties. Most involved in the antibody-antigen reaction are the IgGs (6S- globulins). IgG has a molecular weight of 150,000 - 160,000 and is composed of two halves. Each half is made up of two polypeptide chains, one of molecular weight 55,000 - 60,000 (the heavy (H) chain), and the other of molecular weight 20,000 - 24,000 (the light (L) chain). The chains are held together by disulphide bonds and by non-covalent interaction. The immunoglobulins are considered to be divalent; i.e., there are two regions of the 6S antibody molecule which can combine with an antigenic group. Both combining regions appear to be directed against the same antigenic grouping (Eisen and Karush, 1949; Singer and Campbell, 1953). This combining site is at the free end of the H and L chains. The work of Edelman (1959), Porter (1962) and Feinstein and Rowe, (1965) have shown that the molecule seems to exist in a compact form when free and combined with hapten and in an extended form when combined with antigens in the region of antigen excess. An antibody reacts with the antigen against which it is directed and with certain closely related substances. Very small differences in the structure of the antigen affect the reaction so that the antibody as a rule does not react as well with less closely related substances. Part of this selectivity comes from the requirement that the antigen's

determinant site (that which directs the synthesis of a specific antibody) and antibody have a close steric fit in order that such short range forces as charge interaction, hydrophobic bonds, van der Waals attraction, hydrogen bonds and dipole interactions may be effective (Pressman and Grossberg, 1968). Groupings with shapes different from the determinant group do not fit the antibody site and cannot bind it. This is the basis of immunochemical specificity. The rest of the selectivity rests on the fact that the distribution of groups on the antibody must correspond to the complementary distribution of groups in the antigen such that the short range interatomic forces can be effective. Thus a molecule which fits the antibody combining site sterically but which does not have the correct groups in proper positions will not combine. This combination is necessary for the detection of the antibody-antigen reaction. The most pronounced reaction is the formation of an insoluble complex of the antibody and the antigen which can then be analysed by the techniques developed by immunochemistry. Basically the reaction is chemical. It is specific; entire molecules react; no chemical alteration takes place in the reacting substances after they have combined and the union between antibody and antigen is a firm but reversible one (Pressman, 1953).

C. Carrageenan

(i) Initial studies of carrageenan effect

Carrageenans extracted from Chondrus crispus have been shown to induce connective tissue growth in vivo when injected subcutaneously and to induce the synthesis of mucopolysaccharides and collagen (Robertson and Schwarz, 1953; Slack, 1957; Jackson, 1956a, 1956b, 1957).

McCandless and Lehoczky-Mona (1964) demonstrated that only lambda carrageenan was active in the *in vivo* stimulation of connective tissue growth in guinea pigs. Kappa carrageenan was found to be relatively inactive in this respect. Injection of fragments of carrageenans of differing molecular weights (as determined by viscosity measurements) suggested that a certain degree of polymerization was necessary for the activity of lambda carrageenan.

It may be suggested that the action of carrageenan in producing a granulomatous response may be the result of an immune reaction of the host. Williams (1957) and Burns and Beighle (1960) have postulated that due to the high molecular weight and low rate of diffusion of carrageenan, it may act as a foreign body. A single injection after regression of the carrageenan-induced granuloma results in a heightened response (McCandless, 1967). This suggests an immunological reaction, possibly a hypersensitivity reaction.

Practically no evidence exists that man or experimental animals can develop delayed hypersensitivity to polysaccharides. The reason for this is unknown, but it may be related to their metabolic fate. It has been thought that antigenic activity is in some way related to the digestibility of the antigen. Campbell (1957) has found that non-digestible substances that are not degraded in the tissues do not induce antibody formation; mammalian tissues and cells normally do not contain enzymes capable of breaking down foreign polysaccharides. Allison *et al.* (1966) have shown that carrageenan will disrupt the lysosomal membrane of the macrophage rather than be broken down by the acid hydrolases present in the organelles.

(ii) Chemistry

Carrageenan, a mixture of sulphated (acidic) polysaccharides extracted by hot water from certain members of the Rhodophyta, was originally believed to be found only in the species of the genera Chondrus and Gigartina. Compounds of similar structure have also been found, however, in plants of the genera Furcellaria, Euchœma, Hypnea, Iridaea, and Polyides.

Treatment of whole carrageenan extracts with 0.25 M potassium chloride resolves the polysaccharides into two fractions (Smith et al., 1953). The fraction precipitated by potassium chloride is termed kappa carrageenan. This fraction is composed primarily of chains of α-(1,3)-linked units of carrabiose (4-O-β-galactopyranosyl-3,6 anhydrogalactose) (Anderson and Rees, 1966). This family of molecules contains 29-35% sulphate and 33-44% galactose, varying according to the species from which it is extracted and also from where and at what time the algae were harvested (Black et al., 1965). The galactose is predominantly the D-isomer but the presence of small amounts of the L-isomer has been established for carrageenans from some Chondrus species (Johnston and Percival, 1950; Mori, 1953). Material not precipitated by potassium ions is known as lambda carrageenan by classical definition (Smith et al., 1953). Lambda carrageenan is precipitated by aqueous ethanol into a main component and a polysaccharide containing L-galactose units. Investigation of the main component has shown that it is composed of 3-linked-β-D-galactosyl units and 4-linked-α-D-galactosyl units in alternation (Anderson and Rees, 1966; Dolan and Rees, 1965).

D. Problem

The present studies were conducted to investigate the immunological activity of lambda and kappa carrageenans from Chondrus crispus and other species. Studies centered around the characterization of the antibody response in rabbits to these antigens and the precipitating behavior of these antibodies with other fractions of carrageenans from different species. Basically, the present investigation is the groundwork for further study of the structure of these unique polysaccharides.

CHAPTER II

MATERIALS AND METHODS:

Carrageenan:

Lambda carrageenan, prepared from *Chondrus crispus* and kappa carrageenan prepared from *Gigartina leptorhyncos* and *Chondrus crispus* were obtained from Marine Colloids, Inc., Rockland, Maine. These extracts had been obtained by the potassium chloride fractionation of hot water extracts of the respective alga according to the procedure of Smith *et al* (1953). Analysis of the fractions, as provided by the donor, were:

a. Lambda carrageenan, *C. crispus*, potassium salt

viscosity (1.5% solution, 75°C)	888 cps
3,6-Anhydrogalactose	2-3 %
Ester SO_4^-	36.7 %

b. Kappa carrageenan, *C. crispus*, potassium salt

viscosity (1.5% solution, 75°C)	8.5 cps
3,6-Anhydrogalactose	29.37 %
Ester SO_4^-	30.13 %

c. Kappa carrageenan, *G. leptorhyncos*

3,6-Anhydrogalactose	27.7 %

Polysaccharides were dissolved in 0.9% NaCl which had been adjusted to pH 7.2 with 0.1M NaH_2PO_4 to a concentration of 5 mg/ml. The solutions were dialysed against 0.9% NaCl for 24 hours and sterilized by autoclaving for 10 minutes at 18 psi. For complete solution of the carrageenans the pH of the saline had to be slightly alkaline, particularly in the case of lambda carrageenan.

Nitrogen Analysis

Kjeldahl nitrogen analysis was carried out in the distillation apparatus described by Markham (1942). This method is sensitive in a range of 10 μg N to 100 μg N in protein. The methylene blue-methyl red-boric acid indicator of Kabat (1961) was used in the titrations. The error by this method is approximately $\pm$ 2 μg N with repeated determinations. The Kjeldahl nitrogen determination was used to (1) determine the amount of antibody nitrogen in the precipitates, (2) calibrate the Folin-Ciocalteau protein method with bovine serum albumin as a standard (3) ascertain the presence of nitrogenous impurities in the carrageenan samples.

Immunization

Rabbits were obtained from the Quebec Breeding Farm, Inc., St. Eustache, P.Q. They were maintained on a Purina chow diet. Food and water were constantly provided.

Six male rabbits, approximately 2 kg. in weight, were injected intravenously via the marginal ear vein with 27 mg. carrageenan (either kappa carrageenan from G. leptorhyncos or lambda carrageenan from C. crispus) over a fifteen day period at five day intervals. Responses to a single injection of lambda carrageenan, C. crispus, and to kappa carrageenan, C. crispus were also studied in six additional rabbits. 1 mg. of either carrageenan was administered without adjuvant intravenously via the ear vein. Serum was removed from the animals via the central artery of the ear at day 1, 2, 3, 4, 5, 7, 9, 11, 12, 16, 20, 25.

Antibody assay

Blood was removed from the animal and allowed to clot by standing

at room temperature for two hours; the tubes were sealed and stored overnight at 10°C. The serum was decanted and centrifuged at 10,000 x g for 60 minutes at 4°C to remove free red cells. This was repeated three times. Lipid was aspirated from the top. Where lipid was hard to remove, the serum was clarified by adding 1 volume of cold (4°C) chloroform to 5 volumes of serum. The mixture was shaken vigorously and subsequently centrifuged at 4000 x g for 15 minutes. The clarified serum (upper phase) was decanted and the thick lower phase discarded. This procedure was repeated if the serum persisted in being milky. Serum was kept sterile by the addition of 1 ml. 5% phenol in saline and 0.2 ml. 1% merthiolate in 20 ml. serum to give a final concentration of 0.25% phenol and 1:10,000 merthiolate. The phenol was added dropwise to prevent denaturation of serum proteins (Kabat, 1961). Serum was stored in sealed vials at 4°C.

Pre- and post-immunization sera as well as uninjected control sera were analyzed for antibody titer according to the procedure of Martin (1943). This method involved the setting up of a series of progressive dilutions of serum in order to find the highest dilution at which a precipitate could still be observed when antigen was added. The most important fact was the amount of antigen used in the test. If a fixed quantity of antigen is employed, the proportions of antibody to antigen in the system may vary from one of great antibody excess, through equivalence, to a region of excess antigen, as the serum is progressively diluted. If the amount of antigen used is such that the endpoint of precipitation lies in the inhibition zone (the region of antigen excess) the antibody titer (reciprocal of the dilution factor) observed will depend upon the amount of antigen used in the test as well as on the potency of the antiserum. Thus, the procedure first involved a preliminary titration with a constant volume of antiserum

and progressively decreasing amounts of antigen. The least quantity of antigen which still gives a visible precipitate is then used with progressively decreasing amounts of antiserum and the titer obtained is the reciprocal of the serum dilution; at this level the mixture of antibody and antigen are in equivalent amounts. Serum dilutions of 1:10 to 1:15,000 were employed in conjunction with antigen concentrations 1.0 µg to 500 µg/ml. A total volume of 1.0 ml was maintained; serum sample size was 0.5 ml. The tubes were incubated at 37°C, after addition of antigen to serum, for two hours, then stored in the cold (4°C) for one week. The tubes were inspected daily for evidence of precipitation or turbidity. Appropriate controls were prepared: (1) a control, uninjected serum plus antigen, (2) control, uninjected serum plus 0.15M NaCl, (3) pre-immune serum plus antigen, (4) post-immune serum plus 0.15M NaCl.

Sera were assayed for antibody nitrogen by the quantitative precipitin method of Heidelberger and Kendall (1929) and Heidelberger and MacPherson (1943). Nitrogen analysis of the precipitates was carried out by the Folin-Ciocalteu tyrosine method of Lowry et al. (1951) modified by Kabat (1961). The standard solution was bovine serum albumin. The nitrogen content of the BSA was determined periodically as a check on the calibration of the method. Both pre- and post-immune sera were analysed by this procedure. To determine the entire precipitin curve, increasing amounts of antigen were added to a constant volume of antiserum; a total volume of one ml. was maintained. After addition of antigen, contents of the tubes were mixed thoroughly, then incubated at 37°C for two hours and refrigerated (4°C) for three days.

Maximum precipitation occurred after 48 hours. The precipitates were harvested by centifuging the reaction tubes in the cold at 6,500 x g for 30 minutes. The supernatants were decanted and the precipitates washed with 1.0 ml. cold 0.15M NaCl. This was repeated twice. After centrifugation and washing was completed the tubes were drained until dry and stored in the cold until analysed for protein. After each decantation the supernatants were checked for loose precipitates; if such were observed, the sample was saved and a nitrogen analysis performed on the contents.

Effect of Complement

Maurer and Talmage (1953) had noted that complement in fresh serum rendered antigen-antibody complexes less soluble thus increasing the precipitating power of x quantity of antigen. Complement is also believed to combine with the aggregates thus increasing the quantity of nitrogen in the precipitates. It was of interest to investigate the effect of complement, if any, on this system. Pre- and post-immune sera as well as control (uninjected) sera were tested for their ability to precipitate either lambda or kappa carrageenan from Chondrus crispus under different conditions which are known to influence complement activity. The conditions were: (a) heating the serum at 56°C for 45 minutes, (b) allowing the serum to age for several weeks, (c) precipitating in the presence of 0.1M EDTA. Control sera, anti-lambda carrageenan sera, and anti-kappa carrageenan sera were analysed for precipitating power to their homologous antigens in the presence of the above decomplementary factors.

Precipitation, if any, was analysed as above.

Electrophoresis:

Sera from control, and immune animals, both pre-immune and post-immune, and serum which had been absorbed with homologous antigen were separated by electrophoresis on cellulose acetate (Sepraphore III, Gelman). The TRIS buffer of Aronsson and Gronwall (1957) was used in the electrophoresis procedure. Composition of this buffer is:

10 g. TRIS-(Hydroxymethyl) -aminomethane

1.4 g. EDTA

0.8 g. Boric acid

in 1000 ml. distilled water. This results in a 0.036M buffer, pH 8.6. The stability of the buffer was maintained by preparing it fresh weekly and storing it between runs at 4°C. Resolution was increased if separation was performed in the cold. A two lambda sample of serum was applied at the cathode side and allowed to run for thirty minutes at 300 volts, 15ma. Strips were stained immediately after electrophoresis in Ponceau S (500 mg/ml. in 5% trichloroacetic acid) for 25 minutes. For optimum scanning sensitivity the dyed strips were washed four times in 5% acetic acid, dehydrated in methanol and fixed in methanol-glacial acetic acid (9:1). The strip was stretched over a glass plate and dried at 60°C for 15 minutes. The stained and cleared strip was scanned by an optical densitometer.

Kappa carrageenan, lambda carrageenan and Seakem Type 21 (whole, unfractionated extract of Chondrus crispus) were run on cellulose acetate using the same buffer as above. The samples were applied as spots of about 5 lambda concentration and run at 400 volts, 18 ma for 15 minutes. The strips were stained immediately with 0.1% toluidine blue O (National

Aniline, no. 641) in 3% acetic acid. The strips were prepared as for scanning as before. The carrageenans were also separated by zone electrophoresis in starch gel. Starch gels were prepared according to Smithies (1955) except that the concentration of the starch was 12.5% not 10% A 0.230 M H_3BO_3, 0.0092M NaOH buffer (Smithies, 1955) pH 9.00±.2 was used. The samples were allowed to run for 1½ hours at 200 volts. After separation the gels were refrigerated for 1 hour, inverted from the tray, and sliced through the middle horizontally in order to expose the inner surface where the samples were the most accessible. The two gel slices were stained with 0.1% toluidine blue O in methanol/acetic acid-glacial/water (50/10/50) for thirty seconds and washed in two changes of solvent for two hours.

Gel Filtration:

Sephadex G200 (Pharmacia, Uppsala, Sweden) with a water regain of 20±2.0 mls was soaked in distilled water for 3 days. The fine particles were removed by repeated decantation and the Sephadex carefully packed in a 1.5 x 30 cm. column under 25 mm pressure. When the column was filled, a disc of filter paper was placed at the top of the bed to protect it. The pressure was adjusted to 150 mm and the buffer reservoir attached. The eluting solution was 1.0 M NaCl (Granath, 1963), pH 7.5 with 0.1M NaH_2PO_4. It was saturated with chloroform as a preservative. Carrageenan, 0.2 ml. of a 0.5% solution, was applied to the column. Lambda carrageenan, kappa carrageenan and Seakem Type 21 (whole extract) of Chondrus crispus were applied to the column. 5.0 ml. fractions were collected and analysed for total hexose by the modified Anthrone reaction (Yaphe, 1960).

Preparation of Fluorescein conjugated anti-lambda carrageenan antibody

Antiserum was obtained by multiple injections of lambda carrageenan from Chondrus crispus into four rabbits over a four week period. The titer was maintained by periodic injection. A total of 30 mg. of lambda carrageenan was administered to each animal. Sera were allowed to stand at 4°C for 24 hours and then heated to 56°C for two hours in order to decomplement the serum. Fractionation of the sera was performed according to Coons (1958) with modifications. To fifty mls. of high titer pooled serum 100 mls. of chilled (4°C) 0.85% NaCl were added. The solution was stirred for thirty minutes at 4°C. An equal volume of chilled saturated ammonium sulphate which had been stored over excess ammonium sulphate was slowly added while stirring to the diluted antiserum. The mixture was stirred for 30 minutes at 4°C. The suspension was centrifuged in the cold at 3,000 x g for 30 minutes. The supernatant was discarded; the precipitate was resuspended in cold half-saturated ammonium sulphate and recentrifuged in the cold at 3,000 x g for thirty minutes. This procedure was repeated five times. After the last centrifugation, the supernatant was discarded and the precipitate resuspended in buffered, cold saline, pH 7.5. The suspended globulin fraction was dialysed against 0.85% phosphate buffered saline, pH 7.5 at 4°C with frequent changing of the dialysing solution until the pointwas reached at which no more free sulphatecould be detected. Sulphates were detected by mixing equal portions of a sample of the dialysing fluid and saturated barium chloride; the presence of sulphate will produce a milky, opalescent suspension.

When the globulin solution was free of sulphate merthiolate

powder was added to a final concentration of 1:10,000. The fraction was stored at 4°C in a sealed flask.

Before conjugation of the gamma globulin with fluorescein isothiocyanate, it was necessary to determine the protein content of the fractionated serum. A 0.1 ml. fraction in triplicate was analysed by the Folin-Ciocalteu phenol reagent according to Kabat (1961). Bovine serum albumin was used as a standard.

The procedure of Cherry et al. (1960) was followed in the conjugation of the gamma globulin with FITC. The protein concentration of the gamma fraction was adjusted to 10 mg/ml with 0.85% NaCl. Cold (4°C) carbonate buffer, pH 9.0, equal to 10% of volume of protein solution was added with stirring. The buffered protein solution was chilled and maintained for an hour at 4°C. To each mg. of protein, 0.05 mg. of fluorescein isothiocyanate (E. Gurr, Ltd. C/222) was added with constant stirring. After 45 minutes the conjugated solution was added to a Sephadex column.

The greatest difficulty in the use of the fluorescent antibody technique is the presence of non-specifically staining material which cannot be removed by exhaustive dialysis. Curtain (1961) suggested the use of Sephadex G-25 (Pharmacia Ltd., Uppsala, Sweden) to purify samples. The procedure was carried out according to Curtain (1961) with modifications.

Five grams of Sephadex G-25, superfine, were washed three times with 1 liter of 0.01 M phosphate buffer, pH 7.0, to remove fines. The slurry was poured into a 2 cm. diameter glass tube and allowed to settle under atmospheric pressure. A reservoir of 0.01 M phosphate buffer, pH 7.0, was connected to the column and allowed to equilibrate for 3 hours.

Crude conjugate, 15 ml., was applied to the top of the column, and allowed to filter through the Sephadex. Two fluorescent bands appeared on the column: a weak band travelling with the solvent front containing the fluorescent antibody and a strong band at the top of the column which contained (Curtain, 1961) bicarbonate ions, dioxane, acetone and soluble fluorescein derivatives. The weaker band (the one advancing first) was collected as one fraction after 20 minutes and the column was regenerated by washing the Sephadex for 72 hours with 0.01M phosphate buffer, pH 7.0.

The conjugated fraction was divided into 5 ml. samples, ampouled, and frozen at -20 C.

Cross-reaction studies

Cross-reactions between the antibodies obtained and the carrageenan fractions were investigated by the use of the quantitative precipitin technique described previously in the text. Several carrageenans from different species; carrageenans prepared by two different protocols, and two non-carrageenan substances, were used. The following carrageenan fractions were obtained from Marine Colloids, Inc., Rockland, Maine:

1. Chondrus crispus, lambda carrageenan. This was the immunizing antigen.
2. Chondrus crispus, kappa carrageenan. This was the immunizing antigen.
3. Gigartina pistillata, lambda carrageenan
4. Gigartina pistillata, kappa carrageenan
5. Gigartina radula, lambda carrageenan

6. Gigartina radula, kappa carrageenan
7. Gigartina acicularis, lambda carrageenan
8. Gigartina acicularis, kappa carrageenan
9. Gigartina stellata, kappa carrageenan
10. Type 21 Seakem carrageenan, whole fraction

In addition, the following carrageenans were provided by Dr. A. Haug of the Norwegian Institute of Seaweed Research, NTH, Trondheim, Norway. These had been prepared by fractional precipitation with potassium chloride according to the method of Pernas et al. (1967). The samples provided were:

11. Gigartina pistillata, whole
12. Gigartina pistillata, Fraction 3, (lambda carrageenan)
13. Gigartina pistillata, Fraction 2
14. Gigartina acicularis, Fraction 3, (lambda carrageenan)
15. Gigartina acicularis, Fraction 2
16. Gigartina acicularis, Fraction 1, (kappa carrageenan)
17. Gigartina acicularis, Fraction 3, (alkali-treated lambda carrageenan)
18. Chondrus crispus, Fraction 3, (lambda carrageenan)
19. C. crispus, Fraction 2
20. C. crispus, Fraction 1, (kappa carrageenan)
21. C. crispus, Fraction 3 (alkali-treated lambda carrageenan)
22. C. crispus, Fraction 2, (alkali-treated)
23. C. crispus, Fraction 1, (alkali-treated kappa fraction)
24. Gigartina stellata, Fraction 3, (lambda carrageenan)
25. G. stellata, Fraction 2

26. G. stellata, Fraction 1, (kappa carrageenan)

27. G. stellata, Fraction 3, (alkali-treated lambda carrageenan)

28. G. stellata, Fraction 2, (alkali-treated)

29. G. stellata, Fraction 1, (alkali-treated kappa carrageenan)

The carrageenans above (11-29) were fractionated by potassium chloride. Fraction 1 was the precipitate at 0.0625 M potassium chloride, Fraction 2 was the precipitate between 0.0625 M and 1.5 M potassium chloride, and Fraction 3 was the part soluble in 1.5 M potassium chloride. The recovery in the fractionation procedure was between 84% and 90%. Alkaline treatment was carried out using a 1% carrageenan solution containing 0.1% potassium borohydride and 1 M potassium hydroxide (Smidsrød et al., 1968).

In addition to the above samples, (30) agar (Difco) and (31) chondroitin sulphate (Bovine nasal septa cartilage, sodium salt, Sigma Chemicals) were analysed for ability to react with anti-lambda and anti-kappa carrageenan antibody. The quantitative precipitin curves were determined as described on page 29.

To a standard volume of antiserum (0.5ml.) which had previously been decomplemented, the polysaccharide fractions under investigation were added in quantities of 10 μg to 1,000 μg. Both anti-lambda carrageenan antiserum and anti-kappa carrageenan antiserum to C. crispus, Marine Colloids, were used with the exception of samples 5, 6, and 9 which were titrated with only anti-lambda antiserum. Samples (1-9, 30,31) were titrated against another lot of anti-lambda antiserum which had been obtained prior to that of the serum used in the analysis of fractions 11-29. All polysaccharides were dissolved in 0.15 M NaCl, pH 7.2. The volume of all reaction mixtures was adjusted to 1.0 ml. with 0.15 M NaCl.

CHAPTER III

RESULTS

A. Effect of Complement

Because it had been noted by previous investigators (Maurer and Talmage, 1953) that complement rendered antigen-antibody complexes less soluble, thus increasing the AbN levels for x quantity of antigen, and that complement nitrogen may be incorporated in the aggregates, sera obtained from control, preimmune and immunized rabbits were assayed for any change in nitrogen in the precipitate after incubation with agents that are known to inhibit or destroy complement activity. A volume of 0.5 ml. serum was used in all tests. The total volume in a reaction mixture was 1.0 ml. Incubation at 37°C for 2 hours and storage at 4°C for three days was the standard protocol followed before determination of antibody in the precipitate by the Folin-Ciocalteau procedure (Lowry et al., 1951) calibrated against bovine serum albumin. The agents known to affect complement and used here were: (1) heating sera at 56°C for 45 minutes and cooling to room temperature prior to use, (2) incubation in the presence of 0.1M EDTA, (3) aging the serum for several weeks in the cold (4°C).

Table I gives the results of antisera incubated with homologous antigen in the presence of the decomplementary agents.

Table I

Effect of decomplementary agents on the precipitation of anti-lambda carrageenan serum and anti-kappa carrageenan serum by their homologous antigens

µg AbN precipitated / 0.5 ml. serum

Antigen (µg)	Serum 6.12 (anti-lambda) unhtd	heat	EDTA	aged	Serum 7.10 (anti-kappa) unhtd	heat	EDTA	aged
5	15	18	17	11	--	--	--	--
10	--	--	--	--	22	13	20	15
15	25	20	22	20	--	--	--	--
25	--	--	--	--	46	32	45	30
40	50	48	49	47	--	--	--	--
50	--	--	--	--	54	56	59	51
65	--	--	--	--	80	65	72	68
75	78	60	80	66	86	74	81	75
102	--	--	--	--	98	82	90	80
150	--	--	--	--	95	89	95	88
200	--	--	--	--	95	88	88	86
225	146	140	146	140	--	--	--	--
250	--	--	--	--	94	83	88	85
300	--	--	--	--	90	84	84	80
350	--	--	--	--	80	80	80	72
375	150	142	151	140	--	--	--	--
500	--	--	--	--	72	65	75	59
550	121	104	119	105	--	--	--	--
650	115	84	120	90	--	--	--	--
750	112	78	115	82	--	--	--	--
900	95	60	93	71	--	--	--	--
1000	--	--	--	--	68	59	70	58

There was a decline in antibody nitrogen precipitated after serum had been heated at 56°C for 45 minutes, as compared to the unheated serum. The difference may be due to slight denaturation of the immunoglobulins. Heating and aging for two weeks at 4°C gave comparable precipitin titers; whereas sera treated with 0.1M EDTA showed a very slight decrease in precipitating power, if any, and was comparable in this respect to the unheated sera. Control and preimmune sera failed to precipitate either kappa or lambda carrageenan at any concentration of the antigen. Similarly, no evidence of precipitation was observed in (1) immune sera plus 0.15M NaCl, (2) control sera plus 0.15M NaCl, and (3) preimmune sera plus 0.15M NaCl.

B. Anamnestic response of rabbits to carrageenan injection

Antibody titers of immune sera of rabbits which had been injected with lambda carrageenan from Chondrus crispus (Marine Colloids) or kappa carrageenan from Gigartina leptorhyncos (Marine Colloids) were analyzed by the method of Martin (1943). Analysis of serum from a rabbit which had received the last injection of lambda carrageenan twenty days earlier is tabulated in Table II. These titers are of serum from rabbits which had received a total of 27 mg. carrageenan over a fifteen day period.

Table II

Martin square for direct precipitation test of antiserum: Anti-lambda carrageenan antiserum, 6.20

Serum dilution	Antigen, (lambda carrageenan, Chondrus crispus, Marine Colloids) µg/ml							
	1.0	5	10	25	50	100	250	500
1:1	-	-	+	+	+	+	+	+
1:10	-	-	+	+	+	+	+	+
1:25	-	-	+	+	+	+	+	+
1:50	-	-	+	+	+	+	+	+
1:100	-	-	+	+	+	+	+	-
1:250	-	-	+	+	+	+	+	-
1:500	-	-	+	+	+	+	+	-
1:1,000	-	-	+	+	+	+	+	-
1:2,000	-	-	+	+	+	+	-	-
1:4,000	-	-	+	+	(±)	-	-	-
1:7,500	-	-	(±)	(±)	-	-	-	-
1:10,000	-	-	-	-	-	-	-	-
1:12,500	-	-	-	-	-	-	-	-
1:15,000	-	-	-	-	-	-	-	-

Serum volume: 0.5 ml.
Total volume: 1.0 ml.

(-) no precipitation

(+) precipitation

(±) slight turbidity

It is evident that the endpoint titer of the antigen does not vary with the amount of the antiserum used, indicating that the strength cannot be determined by progressive dilution of the antigen alone.

Serum from rabbit 6.20, anti-lambda carrageenan serum, demonstrates that the titer of the serum is greatest with the 1 st amount of antigen (10 μg in this instance). The titer, 7500, (which is the reciprocal of the serum dilution) obtained at this level of antigen concentration corresponds to a mixture of antibody and antigen in equivalent amounts. Supernatant tests confirmed the presence of antigen in excess and antibody in excess in the respective regions. Failure of precipitation to occur when supernatant was mixed with antigen and with antibody indicated the equivalence zone. The supernatant tests done on serum 6.20 are tabulated in Table III.

Table III

Supernatant tests on Serum 6.20

Serum dilution	Antigen concentration, μg/ml. (Lambda carrageenan, C. crispus, MC) 1.0	5	10	25	50	100	250	500
1:1	ab	ab	ab	ab	ab	ab	Ag	Ag
1:10	ab	ab	ab	ab	ab	(-)	Ag	Ag
1:100	ab	ab	ab	ab	(-)	(-)	Ag	Ag
1:1000	ab	ab	ab	(-)	(-)	Ag	Ag	Ag
1:4000	(-)	(-)	ab	(-)	Ag	Ag	Ag	Ag
1:7500	(-)	(-)	(-)	Ag	Ag	Ag	Ag	Ag

ab - antibody present in excess (detected by adding antigen with a resultant precipitate)

Ag - antigen present in excess (detected by adding antibody with a resultant precipitate)

(-) - no precipitation if either antigen or antibody is added to the serum. Denotes the equivalence zone.

Supernatant expressed in terms of original serum dilution. Volume was 0.5ml. Total volume: 1.0ml.

Table IV

Precipitin titers of rabbit antisera after multiple injections of lambda carrageenan extracted from Chondrus crispus

Rabbits #	Days after last injection								
	3	10	20	50	60	80	100	130	150
1	1000	2000	5000	12000	12000	10500	10000	4000	1000
2	1000	2500	6600	10000	11000	10000	9600	3340	1000
3	1500	2300	8000	11500	10500	10500	9000	2000	1000
4	1500	2000	5500	10600	10600	12000	8500	3500	1000
5	1000	1800	7000	9500	8050	9500	9000	2600	1000
6	1250	1700	7500	10000	9500	9500	7500	4000	1000
7	1600	1500	9000	12500	10000	11050	9500	2000	1000
8	-	1500	6800	7200	7300	7000	6400	2600	1000
*11	-	-	-	-	-	-	-	-	-

*Control animal #11 showed no precipitation at any dilution of both antiserum and antigen.

Table V

Precipitin titers of rabbit antisera after multiple injections of kappa carrageenan extracted from Gigartina leptorhyncos

Rabbits #	Days after last injection									
	3	10	20	30	50	70	110	120	140	150
12	100	200	800	1300	5300	6000	3700	1700	1000	700
13	-	150	750	1500	6000	5500	2900	1600	1000	850
14	200	350	1000	1900	7800	7000	3600	1700	800	700
15	150	260	850	1600	7600	6000	3000	1000	780	500
16	200	200	900	1400	8000	5600	2700	750	700	450
17	150	350	500	1000	6400	5000	2000	1000	700	600
*18	-	-	-	-	-	-	-	-	-	-

*Control animal #18 showed no precipitation at any dilution of both the antigen and the antiserum

Tables IV and V list the antibody titers of the sera of fourteen rabbits immunized with multiple injections of either lambda carrageenan from Chondrus crispus or kappa carrageenan from Gigartina leptorhyncos. Detectable antibody appeared quite rapidly after carrageenan administration; for anti-lambda carrageenan serum a maximum titer was maintained for several months. Kappa carrageenan differed from lambda carrageenan in that the increase in titer was delayed by several days, the titer was not so high, and the maximum level was retained for about a month. After about 150 days without further antigenic stimulation the antibody titers of both lambda and kappa carrageenan antisera were still high (Figure 1).

C. Response to single injection of carrageenan

Intravenous injection of 1 mg. of either lambda carrageenan, Chondrus crispus, Marine Colloids or kappa carrageenan from the same source resulted in the formation of precipitating antibodies (precipitins) as detected by analysis of washed precipitates for AbN by the Folin phenol procedure (Lowry et al.; Kabat, 1961). Sera were analysed by determining the entire precipitin curve over a range of antigen concentrations according to the method of Heidelberger and MacPherson (1943). Tables VI and VII list the antibody contents of sera of six rabbits over a period of 25 days from initial injection. The antibody level reached a maximum about six days after injection; kappa carrageenan was delayed to about the eighth day (Figure 2). The mean anti-lambda carrageenan response was about 22% higher than the antibody level attained by rabbits immunized to kappa carrageenan. The sharp increase in precipitin level and its rapid decline from the sera with time reflects the normal development and decline of antibody levels in serum after an initial antigen dose.

Table VI

Antibody levels in the sera of three rabbits injected with 1 mg. lambda carrageenan, Chondrus crispus, MC

Day	Ab (mg) / ml. serum		
	1	2	3
1	.000	.000	.000
2	.000	-	.000
3	.000	-	.000
4	.322	.350	.321
5	.390	.426	.380
7	.471	.524	.549
9	.500	.549	.581
11	.402	.415	.450
12	.412	.438	.405
16	.252	.274	.298
20	.152	.144	-
25	.150	.142	.100

Table VII

Antibody levels in the sera of three rabbits injected with 1 mg. kappa carrageenan, Chondrus crispus, MC

Day	Ab (mg) / ml. serum		
	1	2	3
1	.000	.000	.000
2	-	.000	-
3	.000	.000	-
4	.000	.000	.000
5	.000	.000	.000
6	.193	.180	.000
9	.325	.350	-
11	.395	.430	.485
14	.379	.400	.415
18	.319	.295	.275
20	.200	.218	.250
25	.070	.095	-

Significantly, in all the animals tested antibody to the respective antigen appeared on the same day. All sera, a total of 44, gave characteristic quantitative precipitin curves.

D. Analysis of precipitin curves

Quantitative precipitin curves of the reaction of the most potent anti-carrageenan sera with either lambda or kappa carrageenan are presented in Figure 3. It is evident from observation alone that the precipitin curves with homologous antigen are of the usual type for polysaccharides (Kabat and Berg, 1953). The lack of a second peak suggests the presence of only one antibody. With both lambda and kappa carrageenan, after maximum precipitation was reached no appreciable diminution of precipitation occurred until several times as much antigen had been added as was required for maximum precipitation; that is, there was little inhibition in the region of excess antigen.

The data used to plot the curves in Figure 3 were tested for their conformity to the two equations where C is antigen added:

$$(1)\quad \text{AbNpptd.} = aC - bC^2$$

$$(2)\quad \text{AbNpptd.} = dC - eC^{3/2}$$

proposed by Heidelberger and Kendall (1935a,b), to describe the course of the precipitin reaction. It was found that if the ratio of the antibody nitrogen precipitated by a given amount of carrageenan is plotted against the square root of the amount of carrageenan added, a straight line relationship results. This is Line II in Figure 4; it graphically represents equation 2. The points plotted in Figure 4 are from data obtained in the titration of serum 3.10 (anti-kappa carrageenan serum to its homologous antigen). If the ratio of the antibody nitrogen precipitated to the amount

of carrageenan added is plotted against the amount of carrageenan added, a linear relationship results if the method of least squares is applied. Graphically, equation 1 is represented in Figure 4 by Line I. Extrapolation of the lines in Figure 4 to the ordinate enables one to determine the constants of equations 1 and 2. The constants a and d equal the intercept on the y axis, and b and e represent the slope of the lines. For serum 3.10 equation 1 becomes: $1.28C - 6.9C^2$ (3) and equation 2 becomes: $1.62C - 3.2C^{3/2}$ (4). Both equations represent the relation between the amounts of antibody nitrogen precipitated and the amounts of antigen (C) added throughout the region of antibody excess up to the point of maximum precipitation. From these equations the maximum amount of precipitable antibody nitrogen (AbN max) in serum 3.10 was calculated by differentiating both equations with respect to C and equating to zero. Equation 3 becomes $1.28/(6.9)^2$ and equation 4 becomes $1.62 - 4.80C^{1/2} = 0$. C max equals the amount of antigen required for maximum antibody precipitation; substituting the values of C max into equations 3 and 4 will give the maximum amount of precipitable antibody N (AbN max). Table VIII summarizes the conformity of the two equations to data obtained with selected serum samples. By inspection of the data, equation 2 fits the data better. Found and calculated values for antibody precipitated are in good agreement; the greatest deviation (0.005 mg) is well within the experimental error of the analytical procedures. The experimentally observed values for C max were not so precisely determined since maximum precipitation occurred over a fairly broad zone of antigen concentration.

TABLE VIII

Conformity of the precipitin data to the empirical equations for the antibody-antigen preciptin reaction.

$$(1) \quad \text{AbN pptd.} = aC - bC^2$$

$$(2) \quad \text{AbN pptd.} = dC - eC^{3/2}$$

Serum	C max, mg calc'd. (Equation 1)	C max, mg calc'd. (Equation 2)	C max, mg expt'l.	Ab max, mg calc'd. (Equation 1)	Ab max, mg calc'd. (Equation 2)	Ab max, mg expt'l.	R
λ-1.9	0.110	0.125	0.100-0.300	0.265	0.324	0.320	2.6
λ-2.7	0.117	0.185	0.150-0.300	0.437	0.545	0.550	2.9
λ-4.10	0.142	0.230	0.100-0.350	0.504	0.625	0.610	2.8
λ-6.8	0.112	0.127	0.100-0.250	0.375	0.392	0.388	3.2
λ-9.8	0.144	0.140	0.100-0.300	0.440	0.425	0.430	2.8
λ-10.10	0.147	0.184	0.100-0.250	0.509	0.565	0.562	3.1
K-7.12	0.112	0.151	0.100-0.250	0.510	0.565	0.565	3.7
K-3.10	0.092	0.114	0.100-0.200	0.347	0.391	0.385	3.4
K-5.15	0.079	0.079	0.075-0.150	0.297	0.290	0.290	3.6
K-8.11	0.073	0.106	0.150-0.300	0.272	0.319	0.325	3.1

Mean value for R (lambda carrageenan antiserum): 2.9 ± 0.2
Mean value for R (kappa carrageenan antiserum): 3.5 ± 0.2
R = mg Ab. max/mg carrageenan (max)
Serum notation (λ-1,9)-λ-anti-lambda carrageenan antiserum
k-1-rabbit number

E. Electrophoretic separations of antisera and of antigens

Figures 5 and 6 illustrate the densitometric patterns obtained after the separation of anti-lambda carrageenan serum and anti-kappa carrageenan serum on cellulose acetate. The pronounced peak in the globulin region of 5A is depressed to a level (5B) comparable to that of the control when the serum had been absorbed by an optimal amount of lambda carrageenan, Chondrus crispus. Similar tracings were obtained with the kappa unabsorbed serum and kappa absorbed serum (Figures 6A and 6B, respectively).

Electrophoresis of both lambda carrageenan, kappa carrageenan and the whole extract, Seakem Type 21, failed to distinguish these polysaccharides from each other. Both zone electrophoresis with starch gel and continuous electrophoresis on cellulose acetate gave unpromising results. Figure 7 shows diagrammatically what was observed on cellulose acetate membranes after electrophoresis of carrageenans.

F. Gel Filtration

Figure 8 demonstrates the elution diagram of carrageenan samples after passage through a Sephadex G200 column. The fractionation range of this column as determined for polysaccharides (Flodin, P., 1962) is of molecules of molecular weights in the range of 1,000 - 200,000. Other investigators have stated the molecular weight of lambda carrageenan as a range of 330,000 to 790,000 and of kappa carrageenan as a range of 260,000 to 320,000 (Smith et al., 1953; Cook et al., 1952). Therefore, it is not surprising that the molecules are eluted in the void volume.

G. Cross reactions

The results of the quantitative precipitin studies of the reaction

of anti-lambda carrageenan serum, Chondrus crispus with various carrageenan samples are shown graphically in Figures 10 to 14, and Table IX.

The reactions between anti-kappa carrageenan serum, Chondrus crispus with various carrageenans are shown in Figures 15 to 18. It is evident that the anti-lambda serum reacts specifically to lambda carrageenan as it will not precipitate kappa carrageenan extracted from the same source (Figure 9). Similarly, anti-kappa serum reacts specifically to kappa carrageenan and will not precipitate lambda carrageenan from the same source. Anti-lambda serum precipitates all lambda fractions from other species in varying degrees but one; the exception is lambda carrageenan prepared from Gigartina stellata by fractional precipitation of KCl soluble portions. Anti-kappa carrageenan serum precipitates all other kappas from other species, to varying degrees (Figure 17). The intermediate fractions vary in their precipitation as do the alkali-treated lambda and intermediate fractions. Alkali-treatment of kappa fractions gives rise to molecules which have great precipitating power with both lambda and kappa antiserum; whether this is a specific phenomenon or the result of non-specific precipitation will be discussed later. As expected, agar (Difco) and chondroitin sulphate failed to precipitate either antiserum (Figure 10).

H. Fluorescent antibody

Work is in progress with this investigation. There is an indication that carrageenan (lambda) is present in cells which have the morphological characteristics of macrophages. Positive conclusions with regard to the ingestion of carrageenan by macrophages using immuno-

fluorescent techniques will have to wait until work with macrophages in tissue culture is attempted.

Table IX

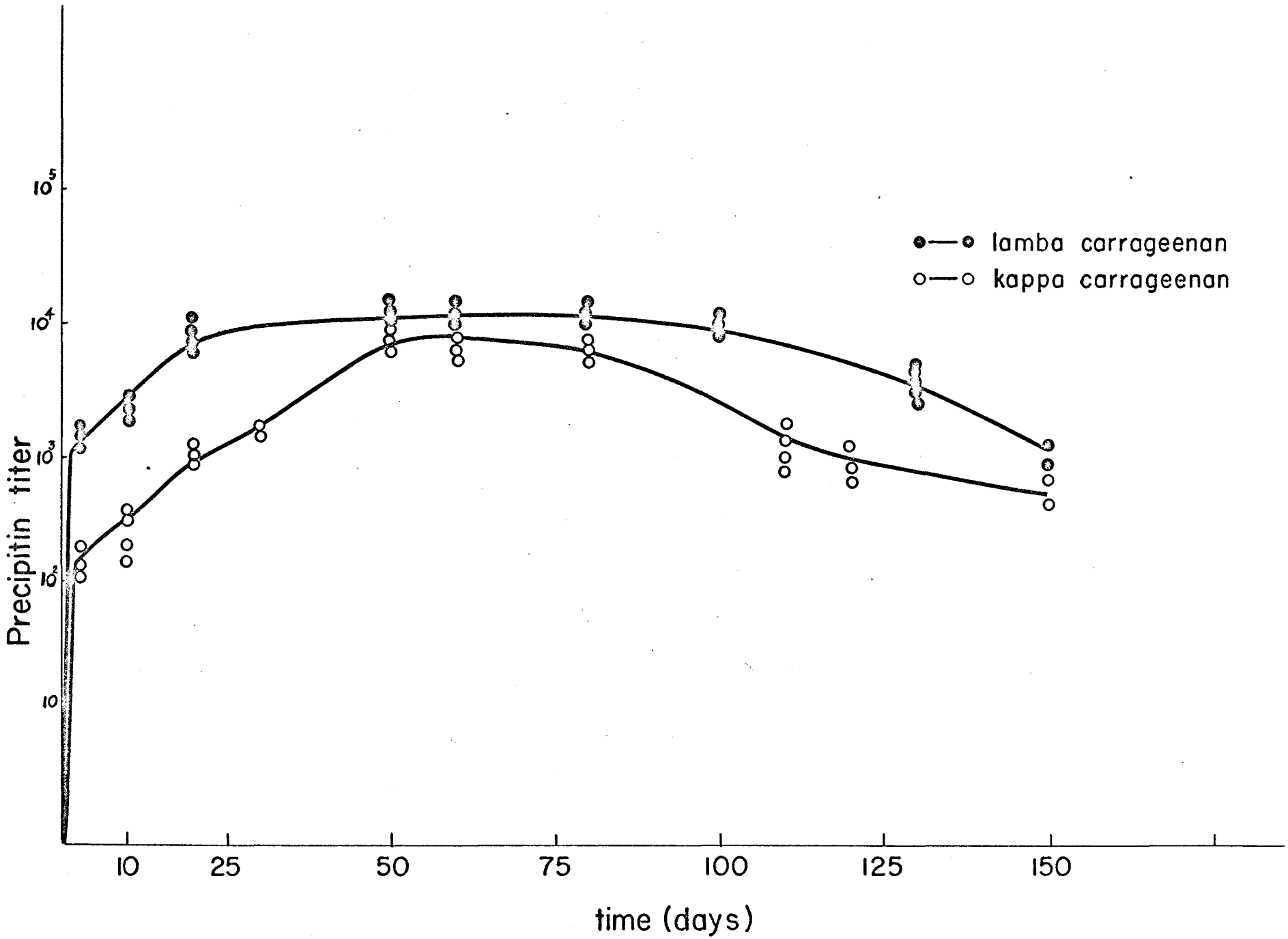
Reaction between carrageenan fractions and anti-lambda carrageenan (C. crispus) serum and anti-kappa carrageenan (C. crispus) serum

Sample No.	AbN pptd µg. anti-λ	% pptd anti-λ	AbN pptd µg. anti-k	% pptd anti-k
1	42	100	-	-
2	3	7.1	-	-
3	42	100	-	-
4	3	7.1	-	-
5	38	90.5	-	-
6	3	7.1	-	-
7	54	128	-	-
8	5	11.9	-	-
9	2	5.0	-	-
10	-	-	-	-
11	-	-	-	-
12	28	57.2	4	8.9
13	41	83.6	4	8.9
14	35	71.4	10	22.2
15	49	100	21	46.7
16	18	36.7	49	109
17	17	36.7	48	107
18	24	48.9	5	9.0
19	14	28.6	45	100
20	7	14.2	31	63.2
21	22	45.0	43	95.6
22	23	46.0	58	129
23	78	159	81	180
24	4	8.2	0	0.0
25	28	58.0	45	100
26	2	4.0	37	82.2
27	59	120	80	163
28	2	40.8	75	167
29	50	100	61	136
30	0	0	0	0
31	0	0	0	0
1A	49	100	4	8.9
2A	4	8.2	45	100
7A	65	133	0	0
3A	27	55.1	5	9.0

(1-10 represents titration with serum 24.26 which was anti-lambda carrageenan)

(1A, 2A, 3A, 7A are the same samples as 1, 2, 3, 7 used with serum 24.26)

Figure 1 Precipitin titers in rabbits injected with either lambda carrageenan from C. crispus or kappa carrageenan from Gigartina leptorhyncos. 33 mg. lambda carrageenan and 29 mg. kappa carrageenan administered over 27 day period.


lamba carrageenan
kappa carrageenan
Precipitin titer
10^5
10^4
10^3
10^2
10
10
25
50
75
100
125
150
time (days)

38

Figure 2 Antibody production in rabbits following single injection (1 mg.) either lambda carrageenan or kappa carrageenan from Chondrus crispus.

(•) lambda carrageenan

(o) kappa carrageenan

Figure 2

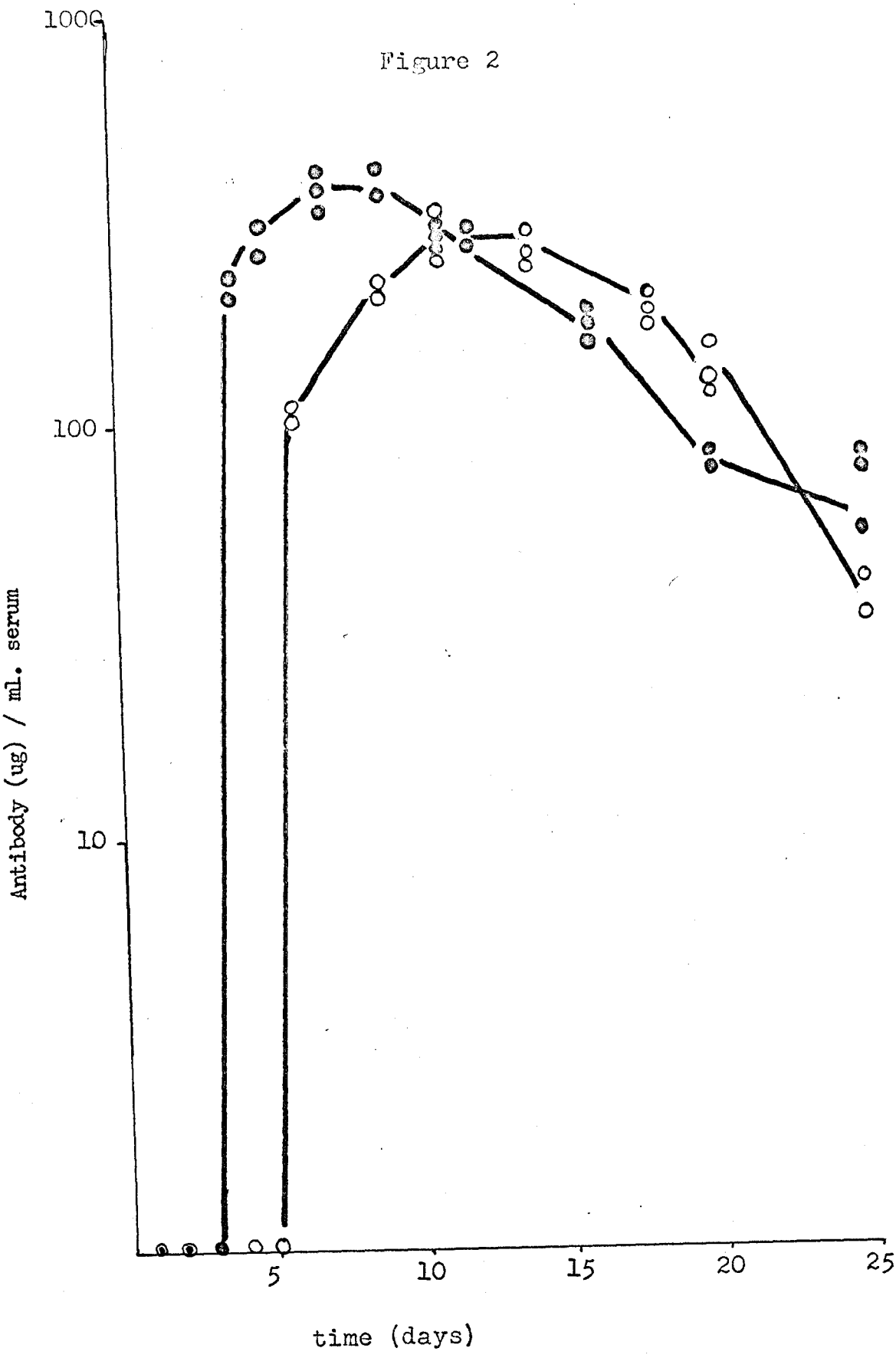


Figure 3 Quantitative precipitin curve of both lambda and kappa carrageenan from C. crispus to homologous antibody.

(•) 2.7:6.8, lambda anti-lambda carrageenan

(o) 3.10:5.15 kappa anti-kappa carrageenan

Figure 3

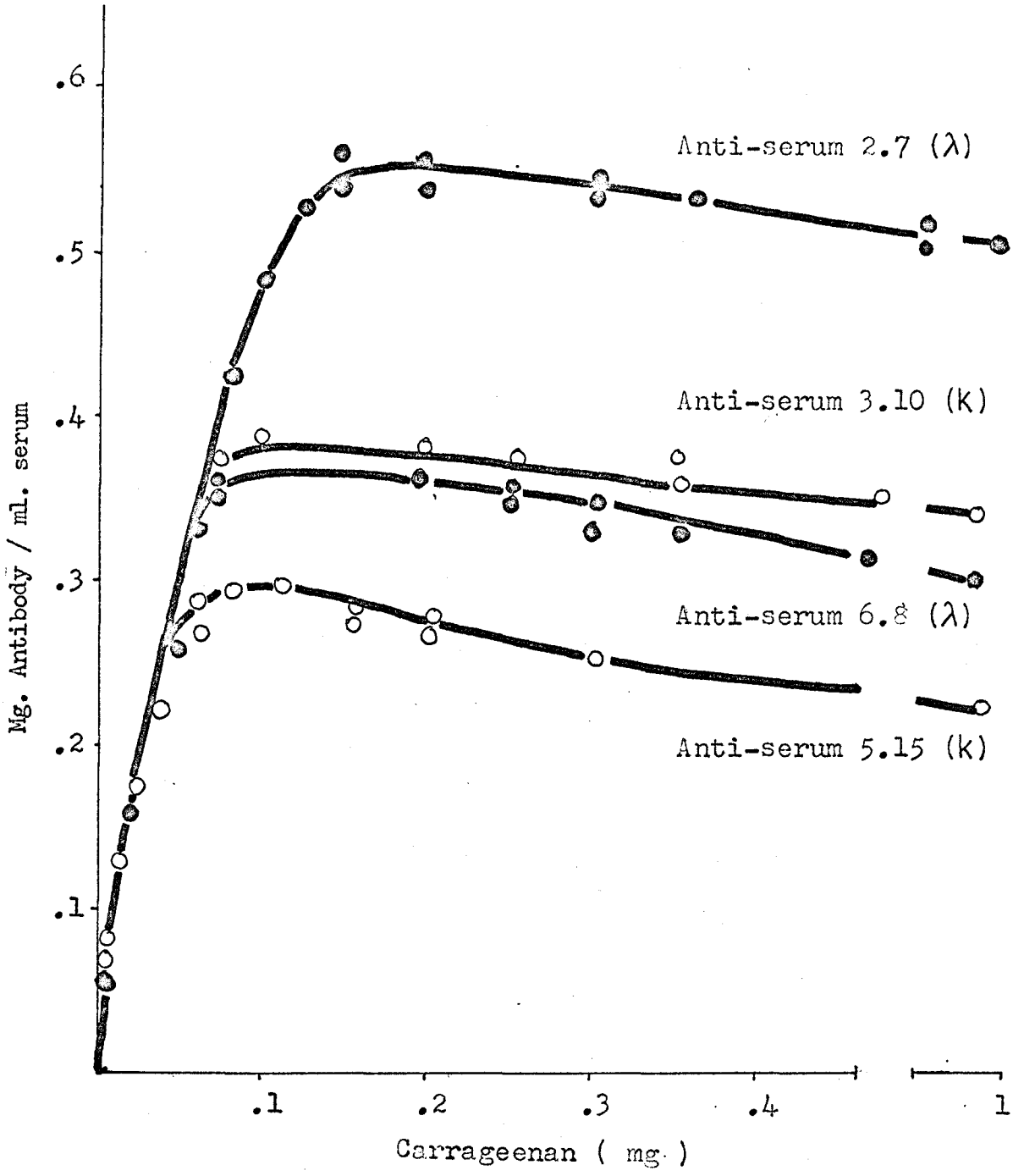

.6
.5
.4
.3
.2
.1
Mg. Antibody / ml. serum
Anti-serum 2.7 (λ)
Anti-serum 3.10 (K)
Anti-serum 6.8 (λ)
Anti-serum 5.15 (K)
.1
.2
.3
.4
1
Carrageenan (mg.)

Figure 4 Quantitative course of the precipitin reaction between kappa carrageenan and rabbit anti-kappa carrageenan (Serum 3.10). Line I plotted as ratio AbN:Carrageenan vs. mg. Carrageenan; i.e., equation 1, AbN pptd. = $aC - bC^2$. Line II plotted as the ratio AbN:Carrageenan vs. Carrageenan: i.e., equation 2, AbN pptd. = $dC - eC^{3/2}$. Line I : $1.28C - 6.9C^2$

Line II: $1.62C - 3.2C^{3/2}$

Figure 4

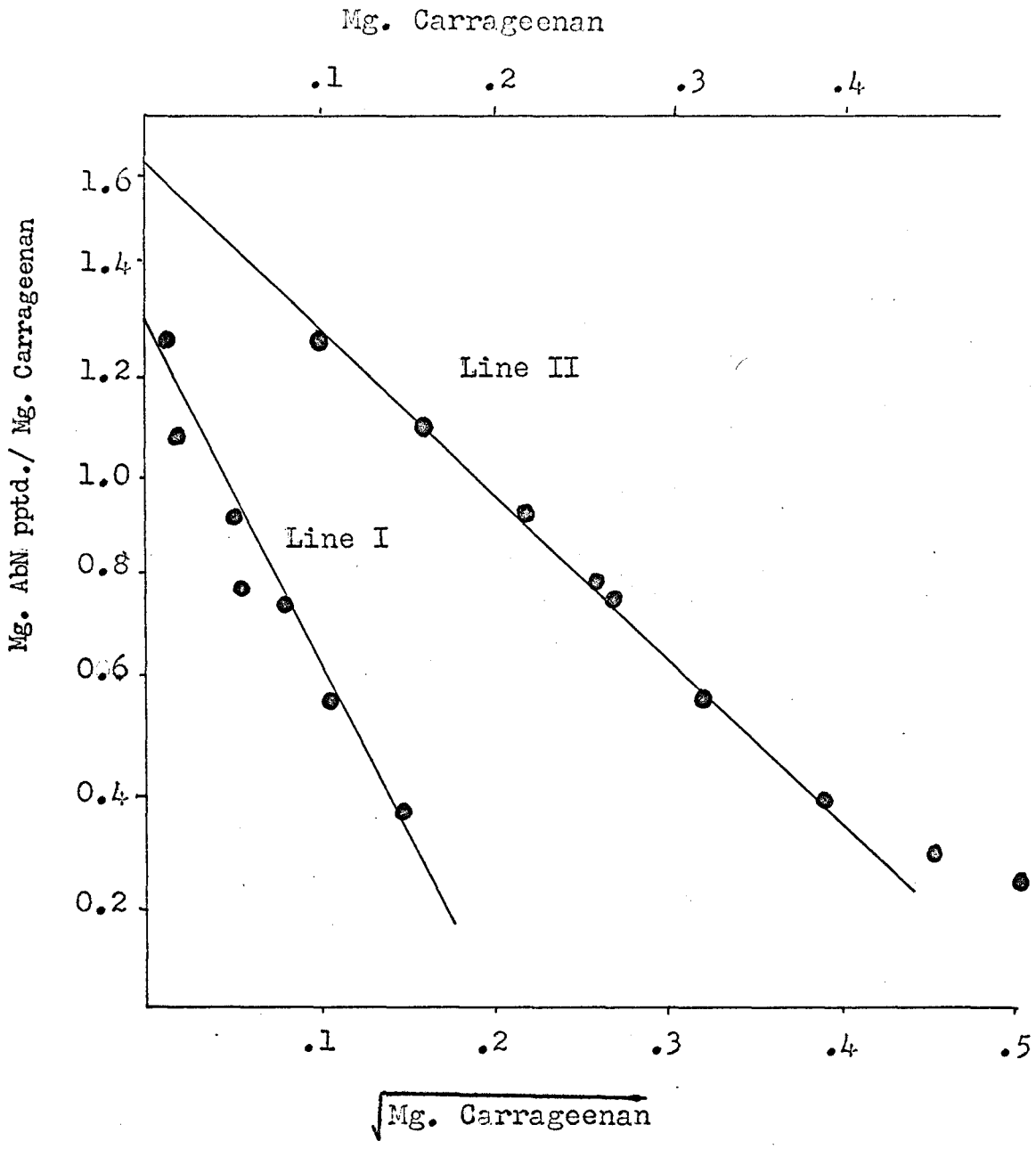

Mg. Carrageenan
.1
.2
.3
.4
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Mg. AbN pptd./ Mg. Carrageenan
Line II
Line I
.1
.2
.3
.4
.5
√Mg. Carrageenan

Figure 5 Electrophoretic patterns of serum before and after absorption of antigen.

Serum 6.12 (anti-lambda carrageenan)

Support: Cellulose Acetate (Sepraphore III, Gelman)

Buffer: TRIS, pH 8.8

Stained with Ponceau S

Figure 5

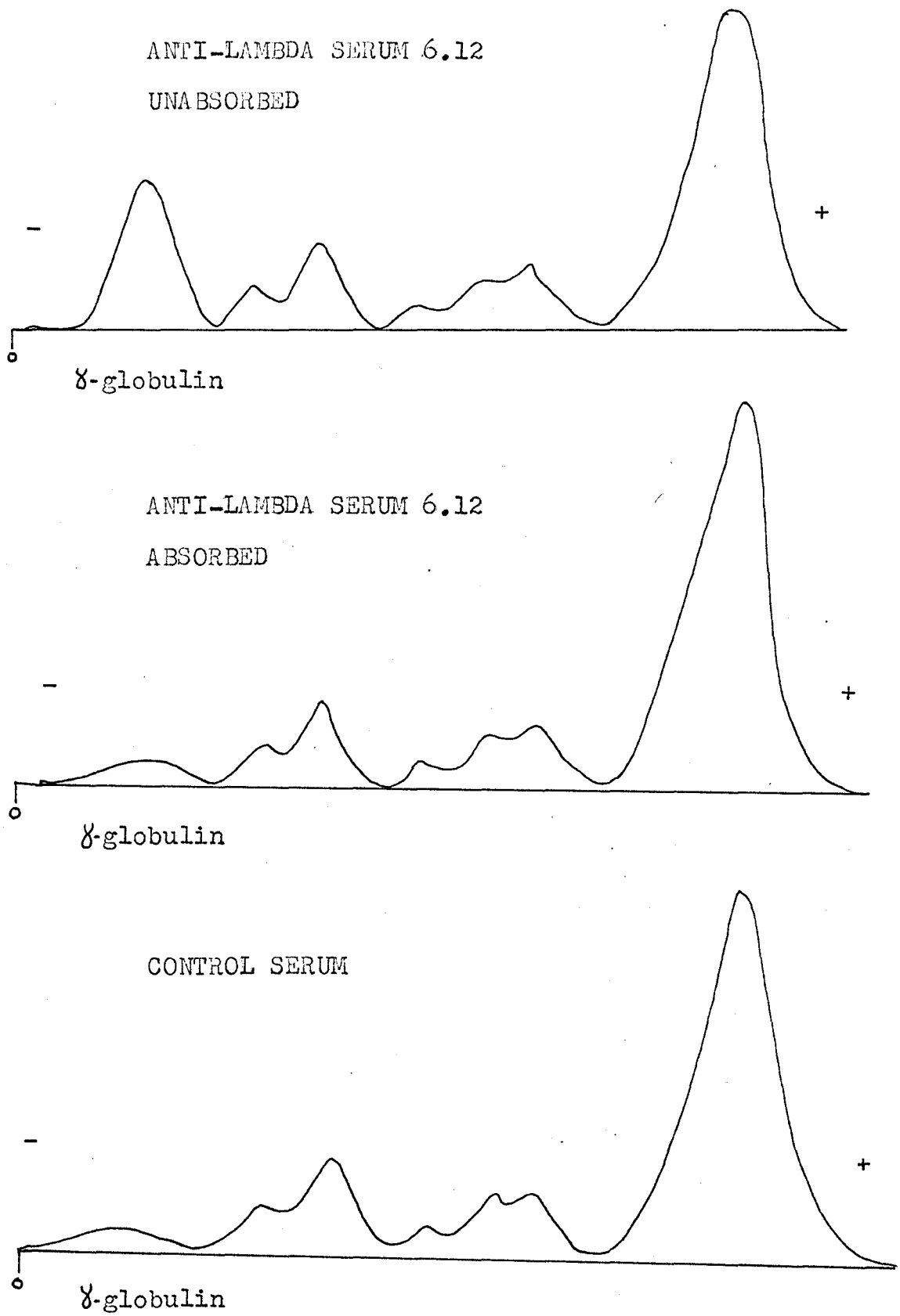

ANTI-LAMBDA SERUM 6.12
UNABSORBED
-
+
γ-globulin
ANTI-LAMBDA SERUM 6.12
ABSORBED
-
+
γ-globulin
CONTROL SERUM
-
+
γ-globulin

Figure 6 Electrophoretic patterns of serum before and after absorption of antigen.

Serum 15.10 (anti-kappa carrageenan)

Support: Cellulose Acetate (Sepraphore III, Gelman)

Buffer, TRIS, pH 8.8

Stained with Ponceau S

Figure 6

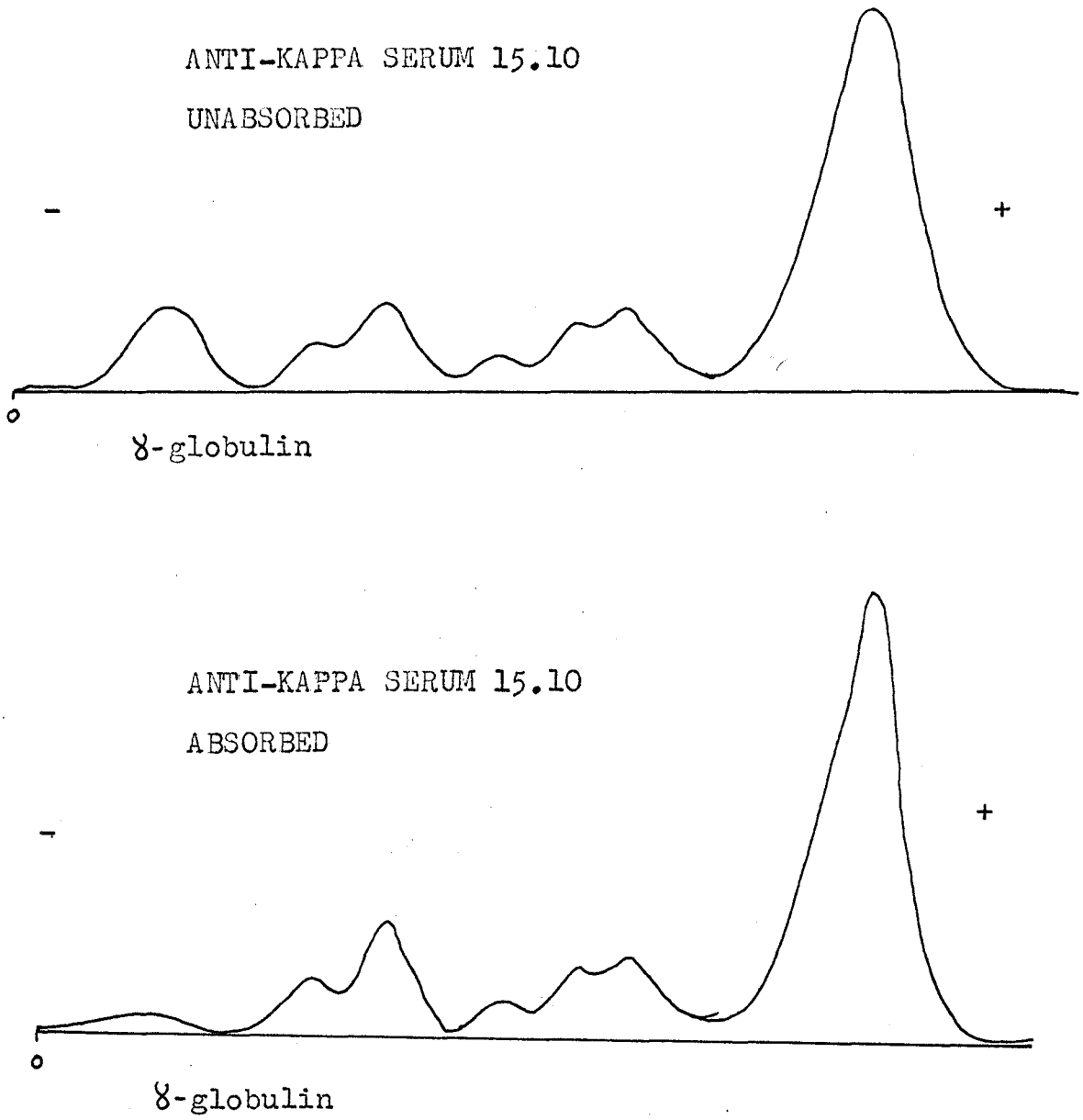

ANTI-KAPPA SERUM 15.10
UNABSORBED
-
+
0
γ-globulin
ANTI-KAPPA SERUM 15.10
ABSORBED
-
+
0
γ-globulin

Figure 7 Electrophoresis of carrageenans on cellulose acetate (Sepraphore III, Gelman). The Buffer was TRIS, 0.036M, pH 8.6. Run at 400 volts, 18 ma for 15 min. Stained in 0.1% Toluidine Blue O in 3% acetic acid.

S_{21} - Seakem Type 21

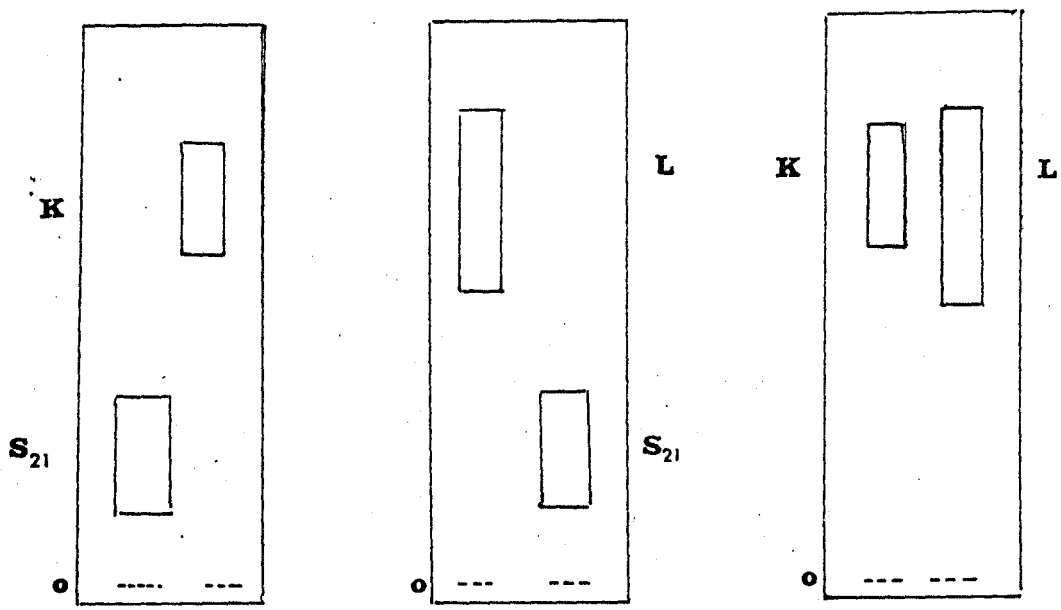
K - Kappa carrageenan, Chondrus crispus (MC)

L - Lambda carrageenan, Chondrus crispus (MC)

O - Site of application

Similar patterns were observed on starch gel.

Figure 7



Electrophoresis of carrageenans on cellulose acetate (Sepraphore III, Gelman). The buffer was TRIS, 0.036M, pH 8.6. Run at 400 volts, 18ma, for 15 minutes. Stained in 0.1% Toluidine blue in 3% acetic acid.

S_{21} Seakem Type 21
K Kappa carrageenan, Chondrus crispus
L Lambda carrageenan, Chondrus crispus
o origin

The same pattern was observed in starch gel.

Figure 8 Fractionation of carrageenans by gel filtration on Sephadex G-200.

Bed dimensions: 1.5 x 30 mm.

Flow Rate: 15 ml/hr.

Eluant: 1.0 M NaCl, pH 7.2.

Carbohydrate content of 5 ml. fractions detected by Anthrone reagent (Yaphe, 1960), absorbance read at 7500 Å.

Figure 8

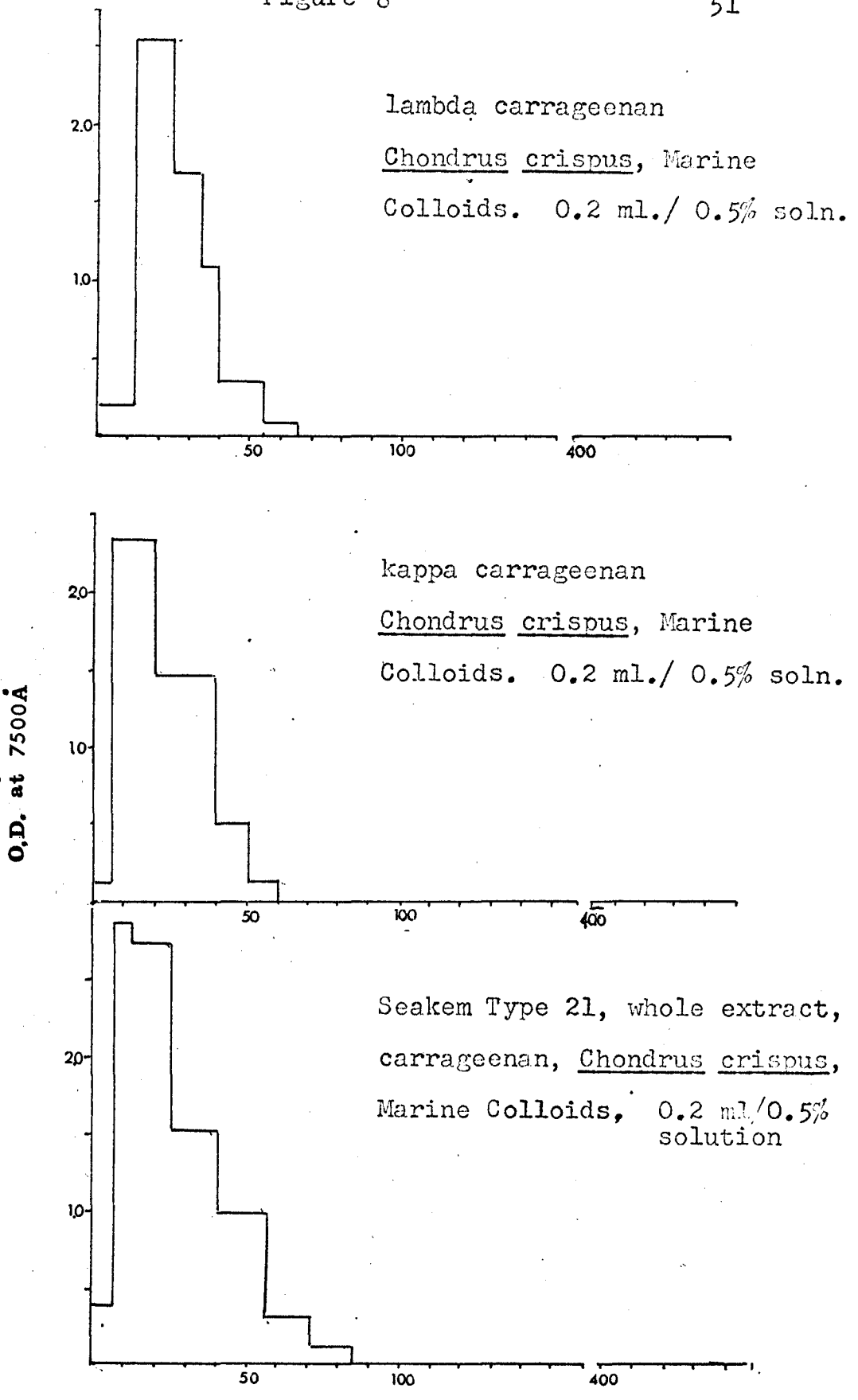

lambda carrageenan
Chondrus crispus, Marine Colloids. 0.2 ml./ 0.5% soln.
kappa carrageenan
Chondrus crispus, Marine Colloids. 0.2 ml./ 0.5% soln.
Seakem Type 21, whole extract, carrageenan, Chondrus crispus, Marine Colloids, 0.2 ml/0.5% solution
O.D. at 7500Å
Elution volume ml.
2.0
1.0
50
100
400

Figure 9 A Reaction between anti-lambda carrageenan antiserum and lambda carrageenan, C. crispus, MC (homologous antigen) and kappa carrageenan, C. crispus, MC

B Reaction between anti-kappa carrageenan antiserum and kappa carrageenan, C. crispus, MC (homologous antigen) and lambda carrageenan, C. crispus, MC

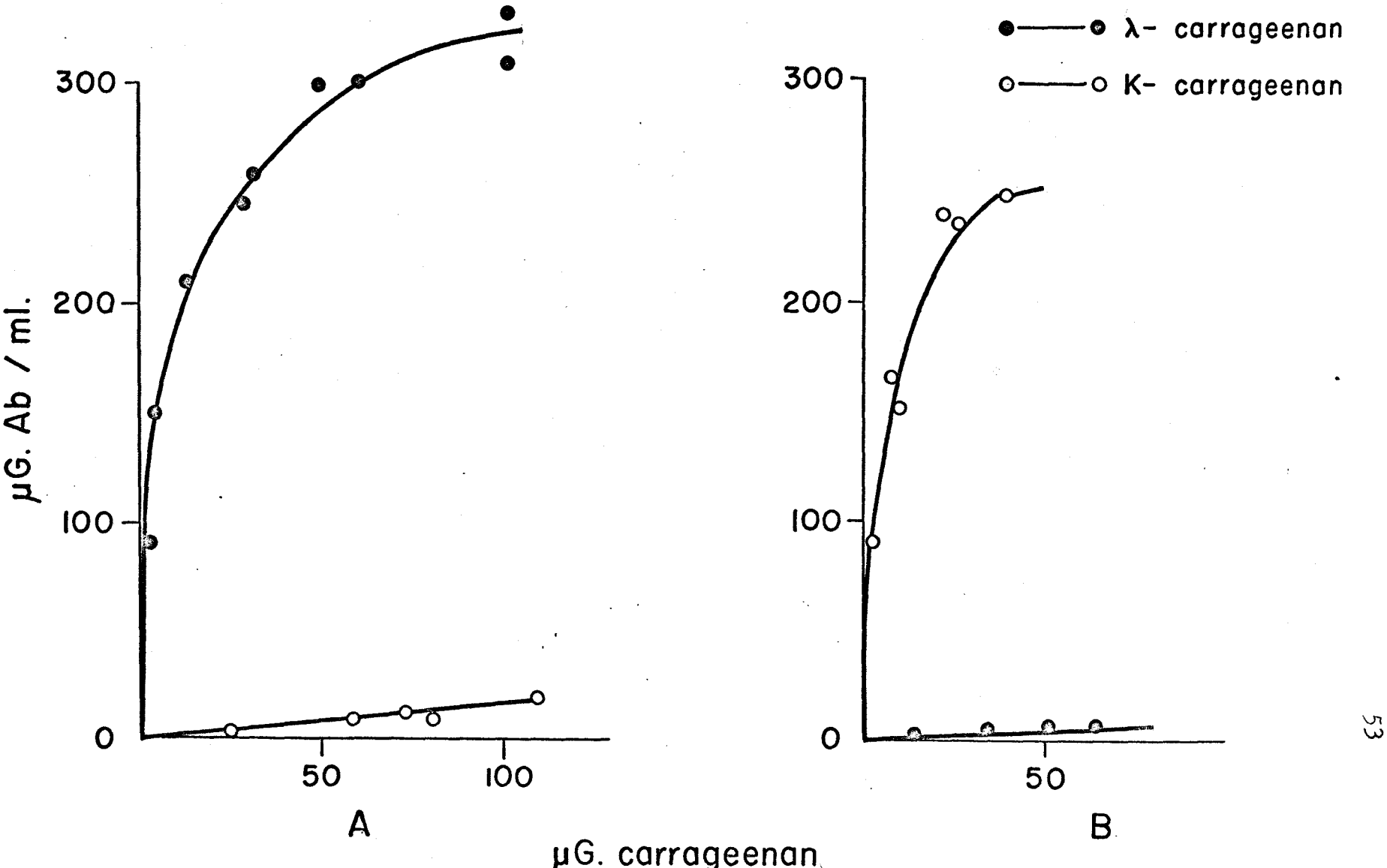

λ- carrageenan
K- carrageenan
300
200
100
0
µG. Ab / ml.
50
100
A
300
200
100
0
50
B
µG. carrageenan

Figure 10 Precipitation of anti-lambda carrageenan serum (C. crispus, Marine Colloids) by:

(●) 1 C. crispus, lambda carrageenan

(⊙) 2 C. crispus, kappa carrageenan

(x) 3 G. pistillata, lambda carrageenan

(▲) 4 G. pistillata, kappa carrageenan

(o) 5 G. radula, lambda carrageenan

(▲) 6 G. radula, kappa carrageenan

(■) 7 G. acicularis, lambda carrageenan

(□) 8 G. acicularis, kappa carrageenan

(▲) 9 G. stellata, kappa carrageenan

(✱) 10 C. crispus. Seakem Type 21, whole extract

(■) 30 chondroitin sulphate

(■) 31 agar

Figure 10

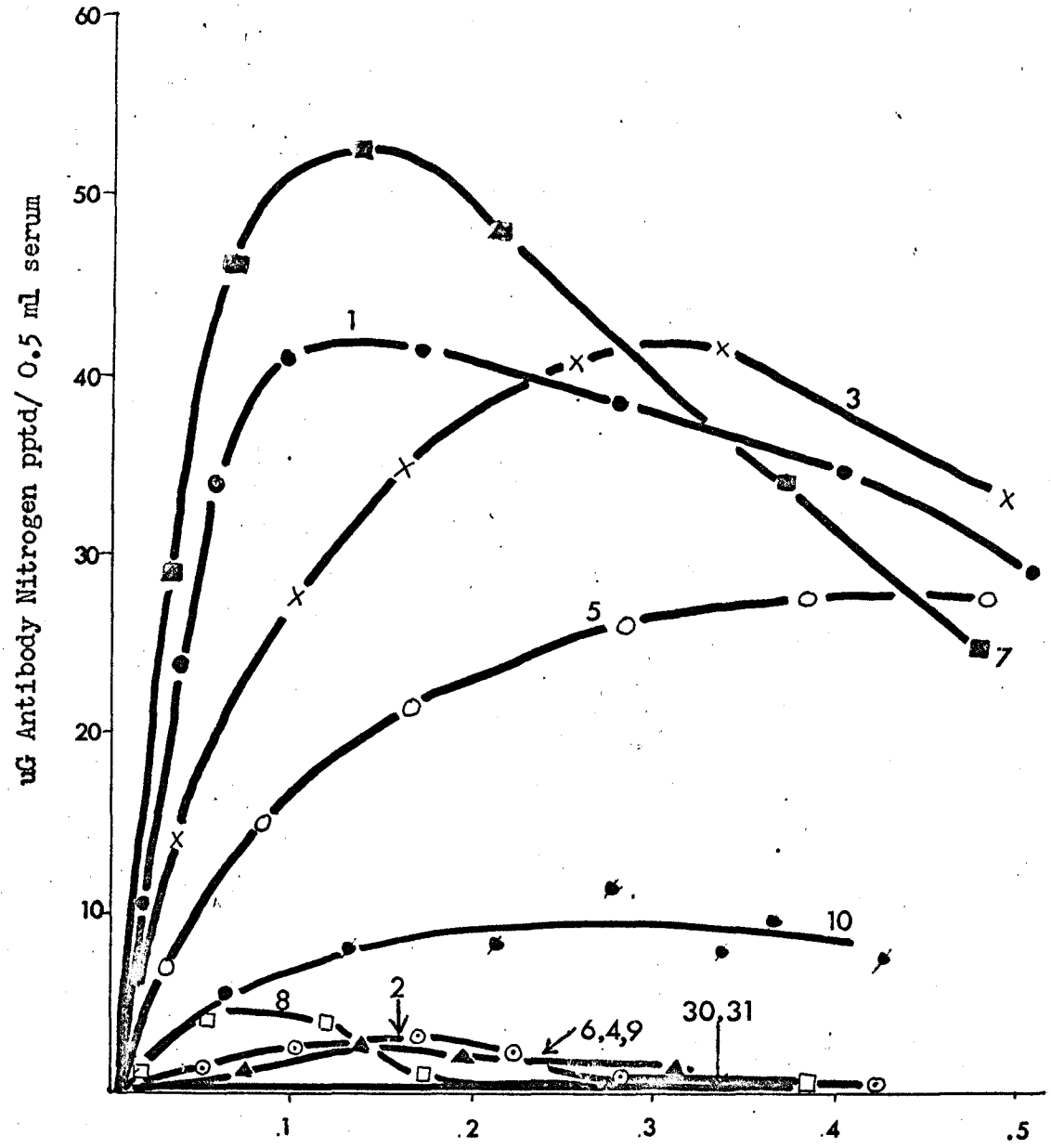

60
50
40
30
20
10
uG Antibody Nitrogen pptd/ 0.5 ml serum
1
3
5
7
10
8
2
6,4,9
30,31
.1
.2
.3
.4
.5
mg. Polysaccharide

Figure 11 Precipitation of anti-lambda carrageenan serum (C. crispus, Marine Colloids) by:

(o) 1 C. crispus, lambda carrageenan

(+) 3 G. pistillata, fraction lambda carrageenan

(x) 7 G. acicularis, lambda carrageenan

(▲) 12 G. pistillata, fraction 3

(●) 14 G. acicularis, fraction 3

(⊙) 18 C. crispus, fraction 3

(■) 24 G. stellata, fraction 3

Figure 11

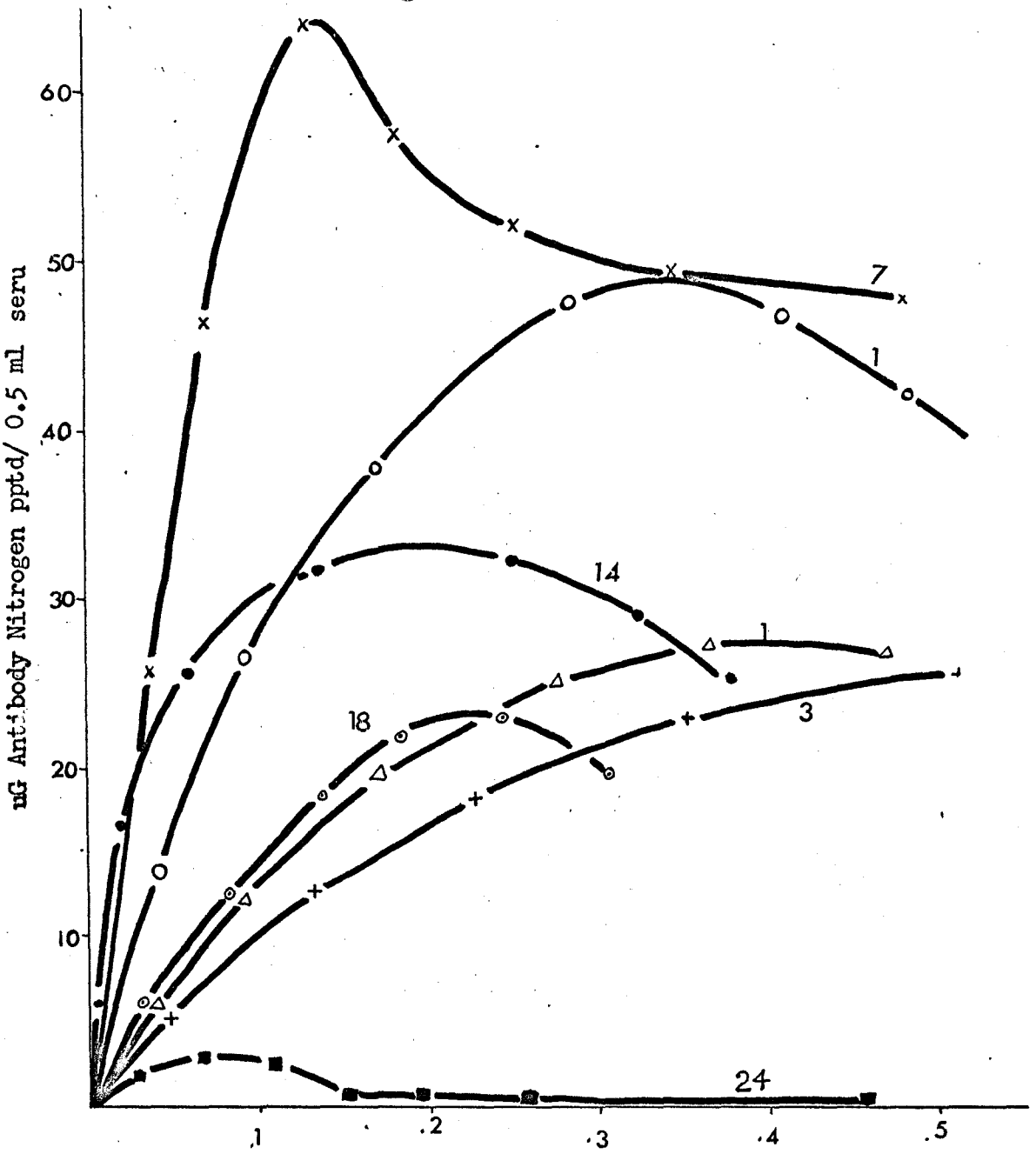

mG Antibody Nitrogen pptd/ 0.5 ml seru
60
50
40
30
20
10
7
1
14
1
18
3
24
.1
.2
.3
.4
.5
mg. Polysaccharide

Figure 12 Precipitation of anti-lambda carrageenan serum (C. crispus, Marine Colloids) by:

(o) 1 C. crispus, lambda carrageenan

(◉) 13 G. pistallata, intermediate fraction 2

(●) 15 G. acicularis, intermediate fraction 2

(x) 19 C. crispus, intermediate fraction 2

(▣) 25 G. stellata, intermediate fraction 2

Figure 12

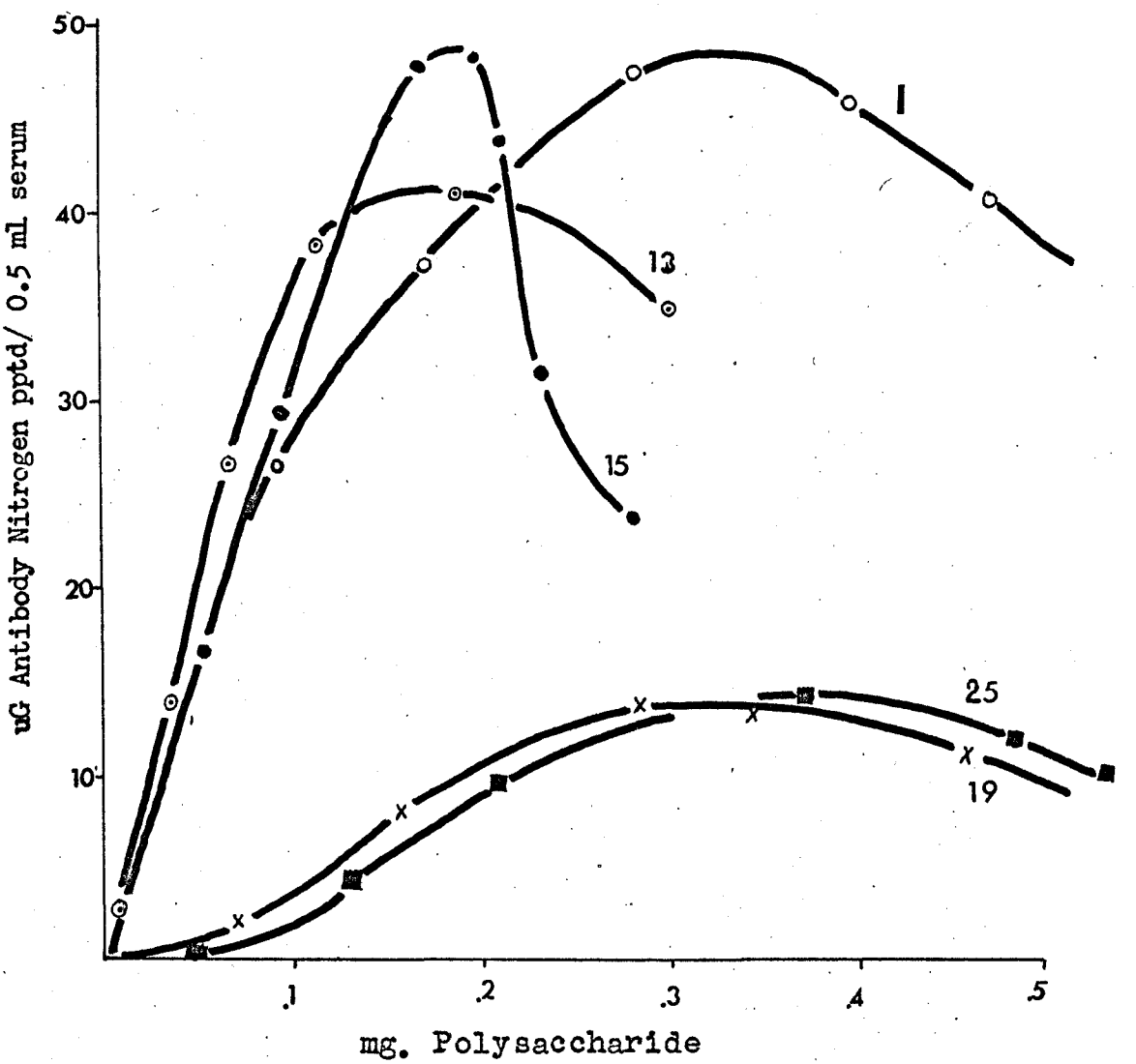

50
40
30
20
10
uG Antibody Nitrogen pptd/ 0.5 ml serum
1
13
15
25
19
.1
.2
.3
.4
.5
mg. Polysaccharide

Figure 13 Precipitation of anti-lambda carrageenan serum (C. crispus, Marine Colloids) by:

(●) 1 C. crispus, lambda carrageenan

(⊙) 2 C. crispus, kappa carrageenan

(o) 16 G. acicularis, fraction 1

(Δ) 20 C. crispus, fraction 1

(x) 26 G. stellata, fraction 1

(▲) 10 C. crispus, Seakem Type 21, whole extract

(□) 11 G. pistillata, whole extract

Figure 13

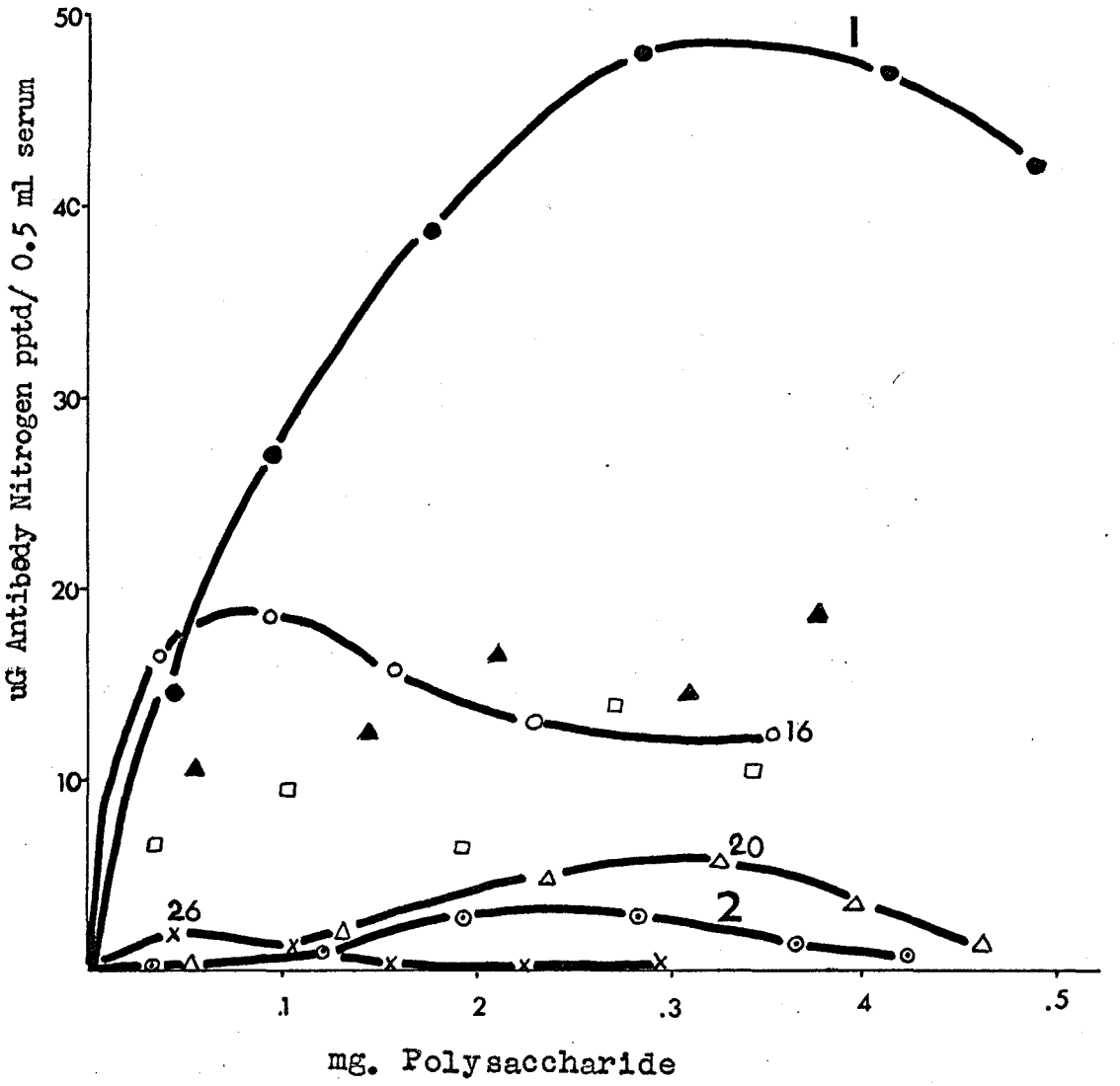

50
40
30
20
10
uG Antibody Nitrogen pptd/ 0.5 ml serum
1
16
20
26
2
.1
2
.3
4
.5
mg. Polysaccharide

Figure 14 Precipitation of anti-lambda carrageenan serum (C. crispus, Marine Colloids) by:

(⊙) 1 C. crispus, lambda carrageenan

(⌀) 17 G. acicularis, alkali-treated fraction 3

(□) 21 C. crispus, alkali-treated fraction 3

(x) 22 C. crispus, alkali-treated fraction 2

(△) 23 C. crispus, alkali-treated fraction 1

(+) 27 G. stellata, alkali-treated fraction 3

(o) 28 G. stellata, alkali-treated fraction 2

(⊙) 29 G. stellata, alkali-treated fraction 1

Figure 14

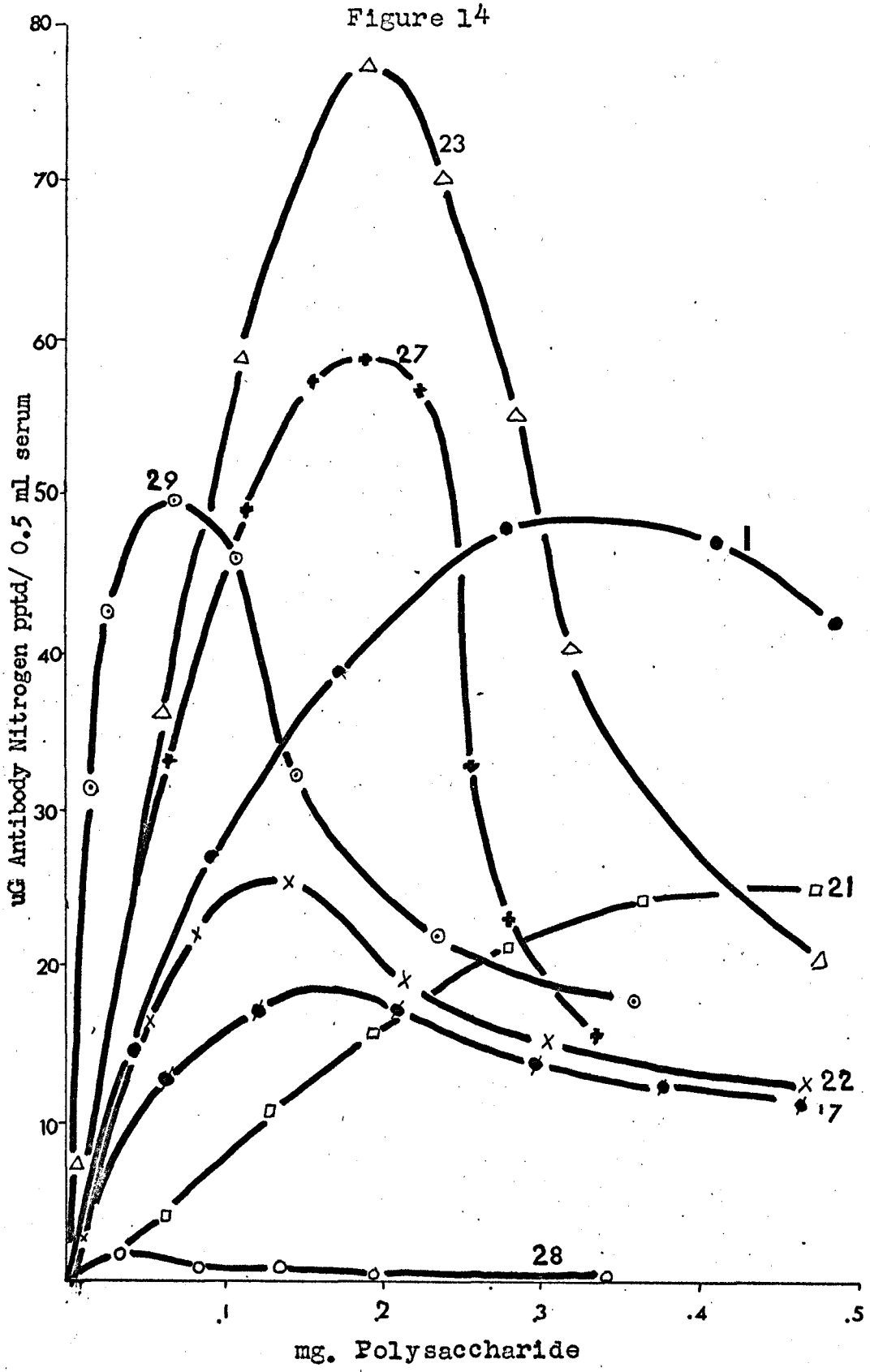

80
70
60
50
40
30
20
10
uG Antibody Nitrogen pptd/ 0.5 ml serum
23
27
29
1
21
22
17
28
.1
.2
.3
.4
.5
mg. Polysaccharide

Figure 15 Precipitation of anti-kappa carrageenan serum (C. crispus, Marine Colloids) by:

(▲) 1 C. crispus, lambda carrageenan

(●) 2 C. crispus, kappa carrageenan

(x) 3 G. pistillata, lambda carrageenan

(ø) 7 G. acicularis, lambda carrageenan

(△) 14 G. acicularis, fraction 3

(o) 18 C. crispus, fraction 3

(ø) 24 G. stellata, fraction 3

Figure 15

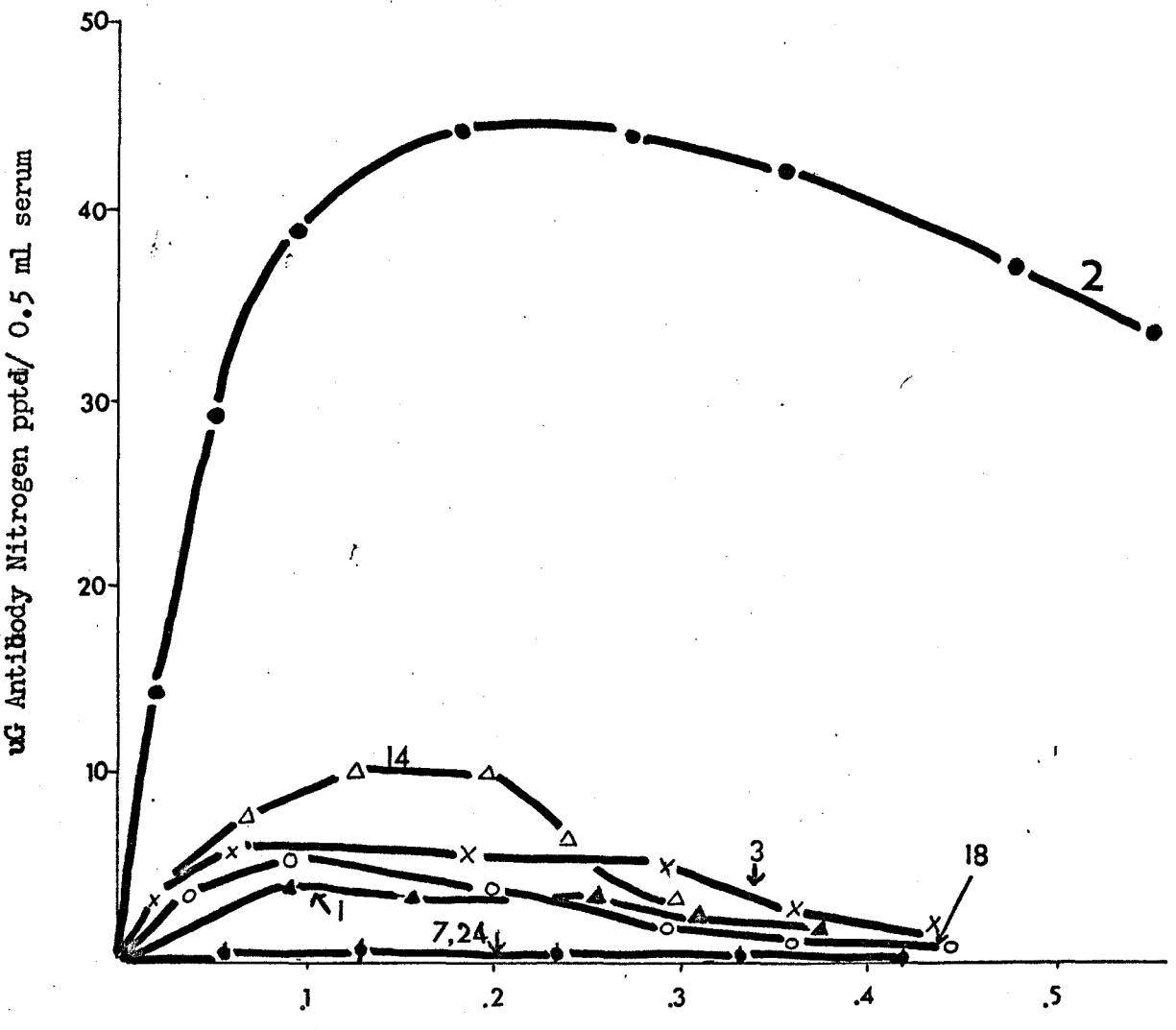

uG Antibody Nitrogen pptd/ 0.5 ml serum
50
40
30
20
10
2
14
3
18
1
7,24
.1
.2
.3
.4
.5
mg. Polysaccharide

Figure 16 Precipitation of anti-kappa carrageenan serum (C. crispus, Marine Colloids) by:

(●) 2 C. crispus, kappa carrageenan

(■) 13 G. pistillata, fraction 2

(+) 15 G. acicularis, fraction 2

(x) 19 C. crispus, fraction 2

(o) 25 G. stellata, fraction 2

(▲) 10 C. crispus, Seakem type 21, whole extract

(□) 11 G. pistillata, whole extract

Figure 16

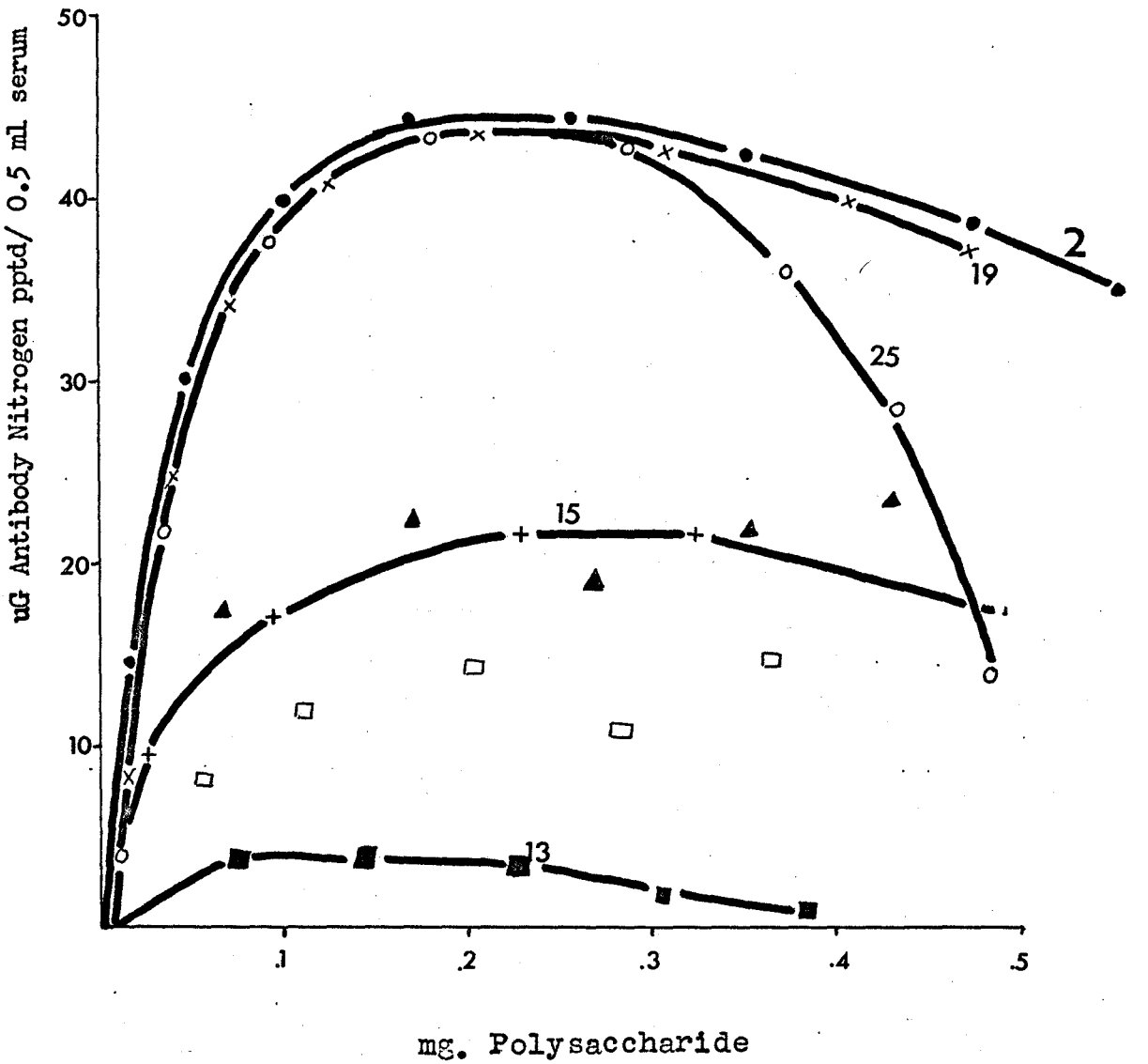

uG Antibody Nitrogen pptd/ 0.5 ml serum
50
40
30
20
10
.1
.2
.3
.4
.5
mg. Polysaccharide
2
19
25
15
13

Figure 17 Precipitation of anti-kappa carrageenan serum (C. crispus, Marine Colloids) by:

(●) 2 C. crispus, kappa carrageenan

(△) 16 G. acicularis, fraction 1

(x) 20 C. crispus, fraction 1

(o) 26 G. stellata, fraction 1

Figure 17

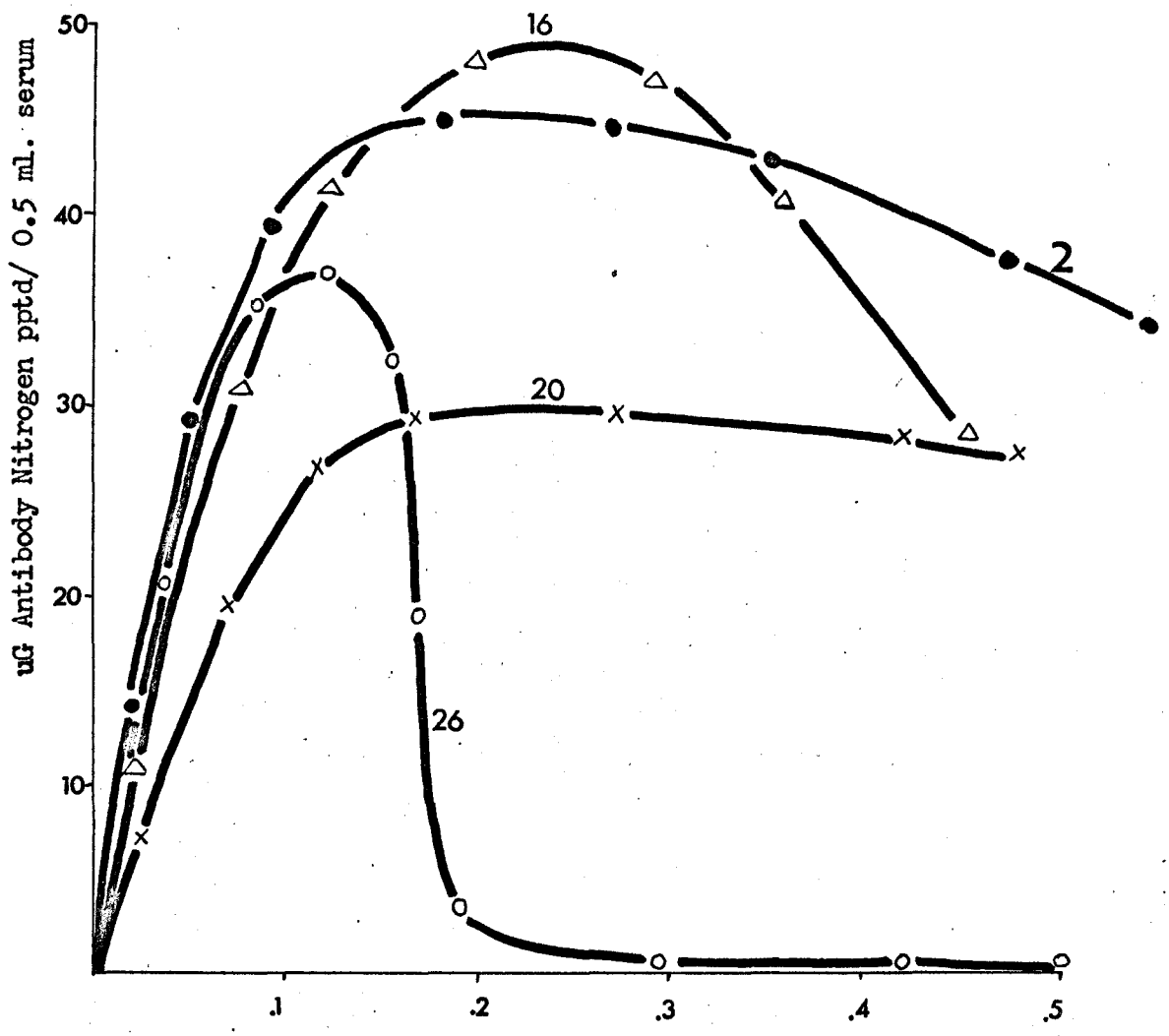

uG Antibody Nitrogen pptd/ 0.5 ml. serum
50
40
30
20
10
16
2
20
26
.1
.2
.3
.4
.5
mg. Polysaccharide

Figure 18 Precipitation of anti-kappa carrageenan serum (C. crispus, Marine Colloids) by:

(●) 2 C. crispus, kappa carrageenan

(x) 17 G. acicularis, alkali-treated fraction 3

(o) 21 C. crispus, alkali-treated fraction 3

(o) 22 C. crispus, alkali-treated fraction 2

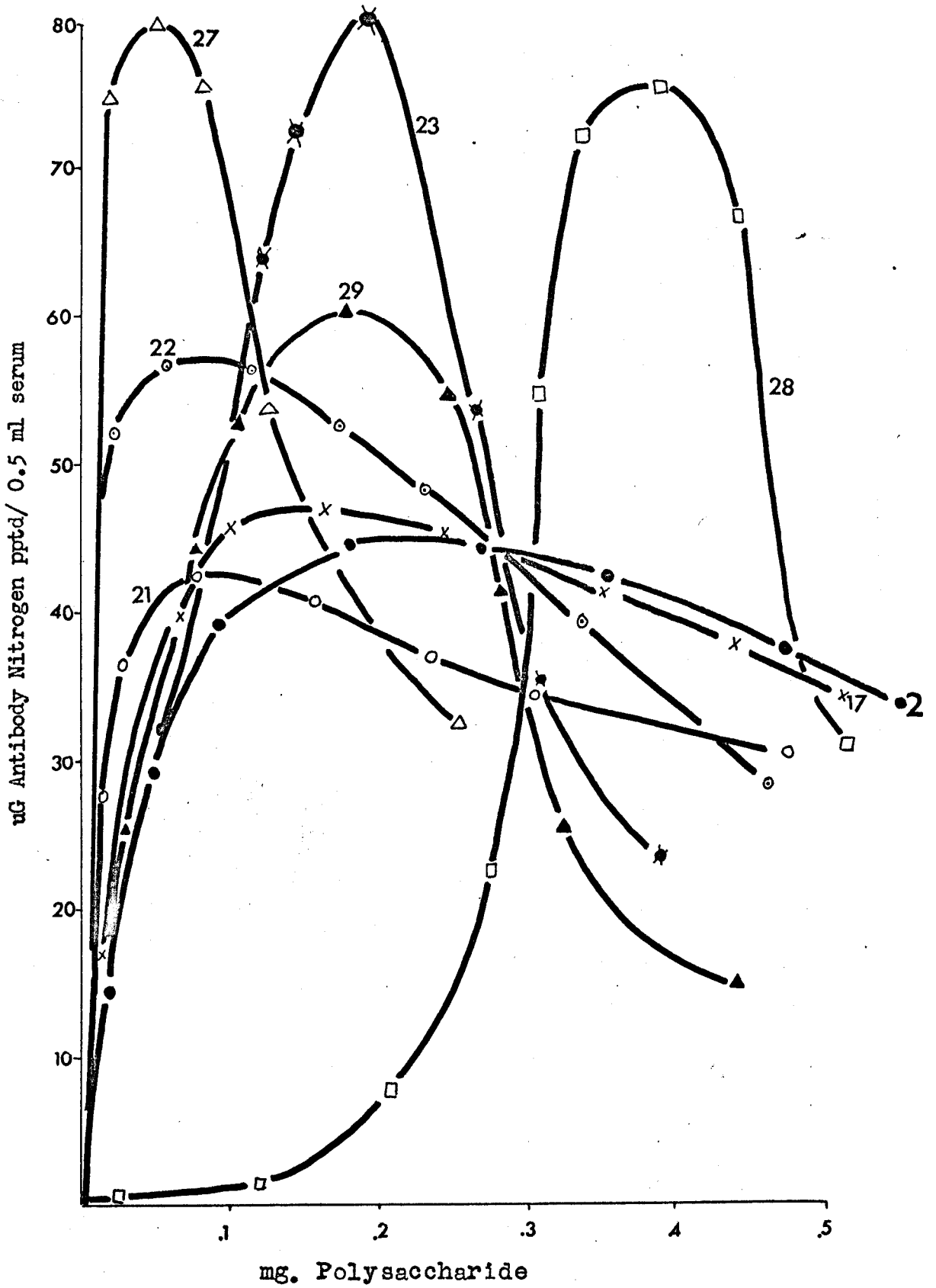
(x) 23 C. crispus, alkali-treated fraction 1

(Δ) 27 G. stellata, alkali-treated fraction 3

(□) 28 G. stellata, alkali-treated fraction 2

(▲) 29 G. stellata, alkali-treated fraction 1

Figure 18


80
70
60
50
40
30
20
10
uG Antibody Nitrogen pptd/ 0.5 ml serum
27
23
29
22
28
21
17
2
.1
.2
.3
.4
.5
mg. Polysaccharide

CHAPTER IV

DISCUSSION

A. Production of antibodies in vivo

The anti-carrageenan reaction is a typical antibody response which can be characterized sequentially by several phases: (a) the latent (inductive) phase which is the interval between the time of antigen injection and the beginning of the exponential rise in antibody concentration in the serum, (b) the log (exponential) phase where the concentration of antibody in the serum increases exponentially, (c) the stationary (plateau) phase where the concentration of the antibody in the blood remains relatively constant, and (d) the decay (decline) phase where the concentration of antibody in the blood decreases exponentially. These phases can be observed in Figure 2. The inductive phase lasted three days for anti-lambda carrageenan antibody and five days for anti-kappa carrageenan antibody; the log phase for about two days and a plateau for about five days. The latent phase, in the opinion of some investigators (McKenna and Stevens, 1957; Svehag and Mandel, 1964; Uhr et al., 1962), is an artifact due to insensitivity of the method for detecting circulating antibody; that is, there should be no lag between antigen stimulation and the beginning of the exponential phase. However, most workers believe (Sterzl, 1960) that the latent phase is real. This lag period may be the time required for the stimulation of non-antibody forming progenitor cells to become geared for the synthesis of a specific antibody protein, a process which requires a finite time.

Immediately after injection antigens appear to be ingested by

phagocytes (macrophages) (Campbell and Garvey, 1963; Thorbecke and Benacerraf, 1962). After subcutaneous injection of carrageenan metachromatic staining with toluidine blue suggests that macrophages are full of carrageenan (McCandless and Lehoczky-Mona, 1964) and ^{14}C-carrageenan studies in this laboratory support this. Kirchheiner (1966) and Kirchheiner and Egeberg (1966) have published electron micrographs of cells believed to be macrophages filled with a material that they suspect is carrageenan. If this material is actually carrageenan, application of the fluorescent antibody techniques should be able not only to detect the presence of the molecule but also to detect whether the molecule is present in its antigenic state.

Long term antibody response is related to prolonged antigen retention by immunologically competent cells (Campbell and Garvey, 1963; Haurowitz, 1960; Richter and Haurowitz, 1960, Richter, Zimmerman and Haurowitz, 1965). After its subcutaneous injection carrageenan has been observed in tissue cells over a period of several weeks (McCandless and Lehoczky-Mona, 1964). If one extrapolates the decay phase plot in Figure 2, a half time (T½) of twenty days is obtained, which could be the result of retention of antigen and release of "antigen stimulator" to antibody forming tissues.

As mentioned previously, for a molecule to be antigenic certain criteria are required. Polysaccharides are no exception. Polysaccharides in their ability to induce antibody formation show species specificities. Purified type-specific carbohydrates from Pneumococcus can induce antibody formation in mice (Avery and Goebel, 1933) and in man (Heidelberger, 1956) but not in rabbits (Avery and Goebel, 1933) or guinea pigs (Maurer and

Mansmann, 1958) unless conjugated with adjuvant. However, Plescia et al. (1964) have found that the charged SIII polysaccharide is antigenic in rabbits after conjugation with methylated bovine serum albumin. This opens the question whether non-antigenic polysaccharides can become antigenic by adsorption to some tissue or serum protein in vivo. Other polysaccharides expressing species differences are levan (Allen and Kabat, 1957), a fructose polymer, dextran (Kabat and Berg, 1953), a glucose polymer, and the Vi antigen of E. coli (Clark et al., 1958), a polymer composed exclusively of N-acetyl-amino-hexuronic acid. These too are found to be non-antigenic in rabbits, but antigenic in man. A primary response in rabbits can be obtained to dextrans and zymosan (a yeast glucan) if administered with adjuvant (Kabat, 1961; Glynn et al., 1954). All polysaccharides discussed above, with the exception of the charged SIII polysaccharide, are uncharged. Carrageenan is an exception also because of its acidic character due to a high degree of sulphation. From the conflicting reports concerning the dependence of charged groups in antigenicity (Sela and Mozes, 1966) it would appear unlikely that the antigenicity of carrageenan or its independence of adjuvant is related to its charge.

The data presented here show that amounts of carrageenan as small as 1 mg. of lambda or kappa fractions injected into rabbits result in the formation of precipitating antibodies (precipitins). The antigenic stimulus (1 mg.) is an amount comparable to that required for immunization with dextran (Kabat and Berg, 1953) and somewhat larger than that used for immunization with pneumococcal polysaccharides (0.05 mg.) (Heidelberger et al., 1946).

Analysis of serum by electrophoresis showed the presence of increased gamma globulin fraction (Figures 5A, 6A) for both antisera. Incubation of the antisera with its homologous antigen and electrophoresis of the supernatant gave a serum pattern which was comparable to the control. The region of the gamma globulin fraction had decreased; the precipitate retrieved after incubation can thus be assumed to be composed of antigen and antibody.

As an antigen, carrageenan fits the requirements quite well. It is not a native molecule even though its chemical structure resembles that of the natural mucopolysaccharides; the molecular size of the molecule is comparable to that of other polysaccharide antigens; the presence of charged groups (half ester sulphates) may or may not be a contributing factor; the presence of alternating linkages results in several conformations for the molecule such that the probability of forming antibodies to such sites is increased; and injection subcutaneously elicits what appears to be a hypersensitive state.

B. Reaction of antibody and antigen in vitro

(i) Effect of complement on the carrageenan-anti-carrageenan reaction

Complement is a component of normal serum. The antigen-antibody combination activates the complement system by converting the first component, C'1, from a proenzyme state to an active esterase (Ratnoff and Lepow, 1963). In turn, other components of the complement system (C'4, C'2, C'3 complex) are activated or consumed until the final step results in an actively lytic substance that attacks the sensitized red cell membrane at strategic sites. The components of complement are proteinacous in nature.

Davies (1963) and (1965) has shown that carrageenan inhibits complement in rabbits and guinea pigs by preventing formation of EAC'1. This could be done either by inactivating directly some part of C'1 or by preventing the sensitized red cell from fixing C'1 by an action on the cell itself. Maurer and Talmage (1953) noted that complement in fresh sera rendered antigen-antibody complexes less soluble, thus increasing the precipitating power of x quantity of antigen. In addition, complement adds to the antibody-antigen aggregate (Weigle and Maurer, 1957). On heating to 56°C and maintaining that temperature for a few minutes, C'1 and C'2 activities are lost. C'3 and C'4 are more resistant; but if heating is continued for 45 minutes most of the C'3 and C'4 activities are lost. By shaking with chloroform C'4 activity is destroyed (Updyke and Conroy, 1956).

In the present experiments, in heated serum, the precipitable antibody to carrageenan was slightly less than in unheated serum; this decrease could possibly be accounted for by slight denaturation of immunoglobulin. Serum which had been allowed to stand for two weeks at 4°C, by which time most of the complement activity would have disappeared, showed little difference from heated serum (Table I). This is in accordance with the observations of Maurer and Talmage (1953) and Kabat and Berg (1953).

Levine et al. (1953) found that EDTA interferes with the uptake of complement nitrogen by aggregates of antibody and antigen by sequestering magnesium and calcium ions. The same authors found that in guinea pig and human serum, uptake of complement nitrogen was inhibited by 0.1M EDTA, whereas rabbit serum complement was unaffected by EDTA. EDTA was without

effect in the present system also.

The absence of a marked effect of carrageenan directly on complement in serum may support the suggestion that carrageenan, like other acidic polysaccharides, adheres to the surface of red cells (Hungerer et al., 1967). If it could be shown that rabbit and sheep erythrocytes can be coated with carrageenan and by this action inhibit complement activity in vivo, it would support this suggestion. Complement can be ignored as a contributing factor in all the work reported here on another basis, as antisera used were always heated at 56°C for 45 minutes and stored at 4°C for at least two weeks before use.

(ii) The precipitin reaction

The antibodies reacted with the carrageenans to give typical quantitative precipitin curves (Figure 3). If the antigen and the antibody are immunochemically homogeneous, or reasonably so (Palmiter and Aladjem, 1968), the precipitin curves of different systems will display certain common features. The initial rise in the amount of precipitate occurs as the amount of antigen added increases over a range more or less coincident with the zone of antibody excess. In antibody excess antigen precipitates antibody almost as if the antibody were univalent. As the proportion of antibody and antigen approaches equivalence there is a change in the nature of the precipitate from a flocculent to a more stable form that is characteristic of precipitates formed with optimal proportions of antigen and antibody at equivalence (Day, 1966). With the formation of the antigen-antibody complexes the aggregates become less hydrophilic and are precipitated out of solution. The precipitates formed

in antigen excess start decreasing in size and strength as total antigen increases. Accompanying this is a breakdown in precipitate composition and a solubilization of complexes. The antigen composition of the precipitates reaches a maximum soon after equivalence and then decreases. A rapid decline is noted in protein precipitin curves; but a slower decline as that observed in the anti-carrageenan precipitin curves, is characteristic of rabbit anti-polysaccharide antibody-antigen systems. This is termed inhibition which may be due, at least in part, to a difference in solubility of various antibodies. The anti-carbohydrate antibodies of horse and rabbit are found primarily in the euglobulin (least soluble) fraction of serum proteins, while anti-protein antibody is found mostly in the pseudoglobulin (more soluble) fraction (Boyd, 1966).

(iii) Molecular weight of antigen in antibody-antigen complex

In the dextran-anti-dextran system, low molecular weight clinical dextran (molecular weight approximately 60,000) caused inhibition in the titration for antibody nitrogen much more rapidly than did native dextrans (molecular weight c. 2,000,000); that is, the clinical dextrans were more effective in inhibiting the reaction per unit weight than native dextrans. Similarly, the mercury monomer of albumin was more effective than the mercury dimer of the same molecule in causing inhibition in the albumin-anti-albumin system (Levine and Brown, 1957). It may be questioned whether the molecular weights for both lambda and kappa carrageenans are similar enough to explain the similarity of the two antigens in their low tendency to inhibit the precipitin reaction in the region of antigen excess, or whether this similarity is due to a fortuitous combination of molecular size and reactive sites per molecule.

A comparison of the kappa and lambda fractions extracted from the same carrageenan sample showed that the lambda fraction had a higher molecular weight: values of 260,000 - 320,000 for kappa carrageenan and values of 330,000 - 790,000 for lambda carrageenan have been estimated by Smith et al. (1955). Expression of the molecular weight of carrageenan as a range reflects the polydisperse nature of the molecules. The electrophoretic separation (Figures 7 and 8) substantiates this, as do the observations of Cook et al. (1952).

In the present experiments, the molecular weights of both lambda and kappa carrageenan in the precipitates were determined by the approach of Boyd and Hooker (1934, 1935).

The authors found that the ratio of antibody to antigen at equivalence in neutral precipitates was influenced by the molecular weight of the antigen. The dependence was not absolute, but a high degree of correlation existed between the two variables. Their argument was based on the fact that in neutral mixtures the surface of the antigen was almost completely covered with a layer of antibody, presumably acting as a chain of four spherical units of molecular weight about 35,200. These authors calculated by spherical trigonometry, that the theoretical relation between ratio by weight of antibody to antigen and the molecular weight of antigen should be approximately:

$$R = \frac{35200\left(2 + \sqrt[90]{\tan^{-1}\sqrt{\tan(3\phi/2)\tan^{3}(\phi/2)}}\right)}{M}$$

where R represents the ratio by weight of antibody to antigen; M is the molecular weight of the antigen; sin ϕ equals p/(1+p); and p equals $\sqrt[3]{352000/M}$. The above equation reduces to: $R = 37800\ M^{-0.8} + 179\ M^{-0.35}$

Using values of R as listed in Table VIII, a value of 380,000 was calculated for lambda carrageenan; and a value of 290,000 was obtained for kappa carrageenan. These values fall within the range proposed by Smith et al. (1955) and Cook et al. (1952). This would also explain the exclusion of carrageenan samples from the column of Sephadex G200 (Figure 8) which has a molecular fractionation range of 1,000 - 200,000. The inability to resolve Seakem Type 21 (the whole, unfractionated carrageenan from Chondrus crispus) into kappa or lambda fractions may be the result of intramolecular bonding between fragments, such that the mixture behaves as one unit. The electrophoretic patterns (Figure 7) support this.

When one considers polysaccharides chain-shaped rather than spherical, one is doubtful of the value for molecular weights obtained by the above method; however, good experimental agreement with the pneumoccal polysaccharides has been obtained (Boyd and Hooker, 1935), even though these are also chain-shaped. Here the fit could be ascribed to the appropriate spacing of determinant groups on the antigen, or perhaps to the effect of steric hindrance to the attachment of antibody. An interference could be more marked in the case of linear molecules than spherical ones.

(iv) Ratio of antigen to antibody at equivalence

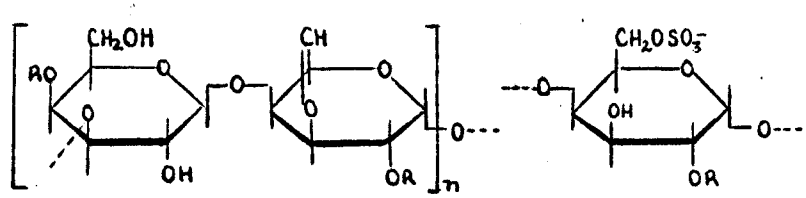
If one accepts the value of 380,000 as the average molecular weight of lambda carrageenan in the precipitate and that of rabbit antibody as 160,000 (Kabat, 1961) the ratio of the weight of antibody to the weight of lambda carrageenan is 0.43. In the region of extreme antibody excess the average value of 3R (Table VIII), the maximum antibody: carrageenan ratio, is 8.7 ± 0.6. Dividing this value by the antibody-antigen ratio gives a value of twenty. This indicates that the composition

of the antigen-antibody complex may be expressed as Ab_{20} carrageenan in the region of extreme antibody excess. At the region of equivalence the composition of the aggregate would be Ab_6 carrageenan (lambda). Similarly, an antigen-antibody ratio of Ab_{19} carrageenan at the region of antibody excess and a value of $Ab_{6.5}$ carrageenan at equivalence was calculated for kappa carrageenan (3R equals 10.5 $\pm$ 0.6; R equals 3.5 $\pm$ 0.2). The molecular composition of human gamma globulin (molecular weight, 160,000) - antibody complexes have been calculated to be HGG_4 anti-HGG in the equivalence zone and HGG_7 anti-HGG in the region of antibody excess (Kabat and Murray, 1950). Mazur and Shorr (1950) calculated the composition of horse apoferritin (molecular weight 465,000) - anti ferritin to be APF_{14} anti-APF and APF_{26} anti-APF in the equivalence zone and zone of antibody excess, respectively. The carrageenan complexes with the respective molecular weights appear to fall within the range of molecular weights of other antigens.

C. Carrageenan

As stated previously, carrageenan denotes a group of galactans extractable by hot water from red algae. Fractionation of the dilute solution with 0.25 M potassium chloride yields at least two components (Smith et al., 1953). The precipitable gel-forming fraction was called kappa carrageenan and the material remaining in the supernatant was termed lambda carrageenan. Treatment of the kappa fraction with warm alkali (Rees, 1961) removes some ester sulphate with the concomitant increase in 3,6-anhydrogalactose, indicating the presence of some β-1,4-linked galactose-6-sulphate. Enzymic studies with kappa carrageenase from Pseudomonas carrageenovora have led to the isolation of a homologous series of

oligosaccharides, based on the disaccharide, neocarrabiose sulphate (4-O-B-D-galactopyranosyl-3 , 6-anhydro-D-galactose) and an enzyme resistant fragment (Weigl et al., 1966; Weigl and Yaphe, 1966). The latter comprises 20% of the polysaccharide, while the former constitutes 80% of the polymer. With alkali treatment the enzyme resistant fragment is converted to an oligosaccharide similar in structure to the enzyme sensitive fraction (kappa) and is also sensitive to kappa carrageenase. Methylation studies have confirmed that all 3-linked units occur as D-galactose sulphated at C-4 and that 4-linked, 3,6-anhydrogalactose units are partially sulphated at carbon 2 (Dolan and Rees, 1965). A small portion of the 1,4-linked-D-galactose units were found to be either sulphated at carbon 6 or disulphated at carbons two and six, and it is these units which give rise to an anhydro-galactose molecule after alkali-treatment. Anderson and Rees (1966) propose that galactose-2,6-disulphate exists as branch units on every fifth kappa disaccharide unit; the degree of branching remains an unsettled question. Rees proposes the following repeating unit for kappa carrageenan:

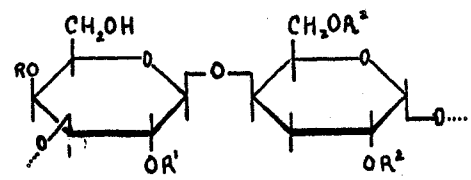


A B

R = H or SO_4^-

Residue B- 2,6-disulphate which will change the form of the 1,4 linked units; alkali-treatment will convert it to a 3,6-anhydrogalactose.

The residual material in the supernatant after potassium chloride precipitation is precipitated by aqueous ethanol; it was termed lambda carrageenan (Smith et al., 1954). Investigation by acetolysis (Morgan and O'Neill, 1959) gave confirmation on the extent of α;1,3 linkages. Rees (1963) showed B,1,4 linkages to 2,6 disulphate and that these comprised 40-50% of the structural units of the polymer. This was concluded from partial mercaptolysis of alkali-treated lambda carrageenan from Chondrus crispus. Treatment with warm alkali resulted in an increase in 3,6-anhydrogalactose and a slight decrease in sulphate esters. In the view of the possibility of side reactions during alkali-treatment such as epoxide formation and of incomplete six-sulphation of the 4-linked galactose units, the estimation of a molecular structure for lambda carrageenan is at best the minimum estimate of the degree of alternation. With this in mind, a hypothetical structure for the lambda carrabiose unit proposed by Dolan and Rees (1965) and Anderson and Rees (1966) is accepted with reservation:



λcarrageenan: R=H;R^1=SO_3^- or H;R^2=SO_3^- or H

third component: R=SO_3^- or H; R^1=H; R^2=SO_3^- or H

The structure of non-modified lambda carrageenan and the third component approximate this formula. Percival and McDowell (1967) suggest that if all the fractions of carrageenans are based on an alternating sequence of 1,3 and 1,4 linked galactose units, fractions differ from one another only in their proportionality of 3,6-anhydrogalactose and sulphate, and in the distribution of these half ester sulphates.

Pernas et al. (1967) concluded from fractional precipitation with potassium chloride that carrageenans could not consist of two distinct components (the kappa and lambda carrageenans of Smith et al., 1954), but rather as a series of molecules of different chemical composition. A fractionation according to the classical scheme (0.125M KCl) was shown to give two heterogeneous fractions, an observation which may explain the results of Black et al. (1965) that kappa and lambda preparations from different carrageenans were of different chemical compostion. Rees (1966) has suggested that lambda carrageenan is a biological precursor of kappa carrageenan, and that the differences observed between fractions may represent the states of transition between lambda and kappa (Figures 10 to 18).

D. Cross-reactions and other problems

While antibodies generally react only with the antigen used in the immunization (the homologous antigen), reactions do occur with substances other than the homologous antigen. These cross-reactions are due to structural similarities between the antigens concerned. As defined by Kabat (1961) the concept "cross-reactivity" applies to serological relationships between different single antigens which presumably possess similar structural groupings within their molecules. Certain fractions of carrageenans from different sources appear to show homogeneity of immunochemical specificity.

This is interesting in that the samples used to immunize were not 100% pure; the lambda carrageenan preparation had 2-3% anhydrogalactose. By reference to Table IX (Figures 10 and 11) it can be noted that anti-lambda carrageenan serum to Chondrus crispus (MC) will precipitate with lambda fractions of Gigartina acicularis (7) (14), Gigartina pistillata (12) (3), Chondrus crispus (18), and Gigartina radula (T); however, fraction 3, lambda, of Gigartina stellata (24) did not precipitate anti-lambda serum. The intermediate carrageenan fractions (insoluble between 0.0625M and 1.5M potassium chloride) showed precipitation with the same serum to varying degrees; G. acicularis (15) being the most active in this respect. G. acicularis (7) obtained from Marine Colloids precipitated anti-lambda carrageenan antibody in excess of that precipitated by the immunizing antigen, to the extent of 133% to 128%, for two serum samples (Figures 10 and 11). If one assumes that the antibody is directed towards a common determinant group, but that part of the antigen-antibody complex may be soluble, this is not surprising.

Inspection of the curves of G. acicularis, intermediate fraction (15) and G. acicularis, lambda (7), would make it appear that these molecules are quite similar in their tendency to precipitate the anti-lambda serum. The sharp rise, small region of equivalence and rapid decline of the precipitin curve is reminiscent of that for small molecular weight compounds (Luderitz, Staub and Westphal, 1967).

The kappa fractions (insoluble at 0.0625 M potassium chloride) precipitated negligible amounts of anti-lambda antibody; an exception was G. acicularis (16) which is believed by some to contain a heterogeneous mixture of lambda and kappa fractions (Smidsrød et al., 1968). This

precipitation may be the result of contamination with lambda, as there was lack of precipitation between kappa carrageenan and lambda antiserum and between lambda carrageenan and kappa antiserum (Figure 9). Any slight precipitation of kappa carrageenans may be accounted for by the presence of small amounts of homologous determinant groups or to the presence of a secondary determinant site - "the kappa determinant" - on the lambda molecule used for immunization. This is not unlikely as lambda carrageenan used for the production of the antiserum contained about 2-3% 3,6-anhydrogalactose; the latter may elicit an antibody response over an extended injection schedule. It has been observed that the precipitating power of C. crispus (2) kappa carrageenan to anti-lambda serum increased with time until a maximum of about 10% was reached from whence it declined. This is similar to the observations of Mage and Kabat (1963) in the immunizing schedule for anti-dextran (α-1,6 linkages); they observed the appearance of antibodies to α-1,4 linkages after several months of injections after which the level of this antibody declined. It is well known that antibody populations become more heterogeneous with time. It is therefore not only possible but most likely that there are at least two immunodominant sites that is, one which is unique to the lambda fraction, and one which is unique to the kappa fraction.

Chondrus crispus, fraction 3 (18) precipitated 48.9% anti-lambda serum; only 9.0% of anti-kappa serum was precipitated by the same fraction. Upon alkali-treatment with potassium hydroxide (1 hr. at 100°C in the presence of potassium borohydride) the precipitating power decreases slightly in anti-lambda serum from 48.9% to 45.0% but increases markedly the ability to precipitate anti-kappa serum from 9.0% to 95.6%. Rees (1963) and Dolan

and Rees (1965) have demonstrated that treatment with alkali results in an increase in 3,6 anhydrogalactose with a concomitant decrease in sulphate. In this sample, the 3,6 anhydrogalactose content increased from 4.9% to 22.0%, but a stoichiometric decrease in sulphate was not observed (data provided by A. Haug, Trondheim, Norway). Because all kappa fractions (insoluble at 0.0625M potassium chloride) contain a high percentage of 3,6 anhydrogalactose (approximately 29-33%) and because alkali-treatment of all samples results in an increased precipitation of that serum, one could propose that the 3,6-anhydrogalactose molecule is intimately involved in the "kappa determinant".

One feature of the lambda component from C. crispus is the presence of a sulphate half ester group at position 2 in the β-1,4-linked galactose unit (that is, the unit which becomes 3,6-anhydrogalactose after alkali treatment). Alkali-treatment of fraction 3 of C. crispus (18) increases 3,6-anhydrogalactose content but should not remove the sulphate on carbon 2 (Rees, 1963). This resultant fraction (alkali-treated lambda, 21) was 92% as active as the untreated sample (18) in precipitating anti-lambda serum. Retention of sulphate on C-2 may have retained the molecule's affinity for anti-lambda serum. Pernas et al. (1967) noted that the marked physical difference between samples with similar 3,6-anhydrogalactose content could be attributed to different distributions of 3,6-anhydrogalactose units among the molecules; similarly, the distribution of half ester sulphate groups may vary (Painter, 1966). The presence of the 3,6 anhydrogalactose in conjunction with the lack of C-2 sulphate would satisfy the requirements of a "kappa group" and absence of a "lambda group". The intermediate fraction (19) of C. crispus precipitated 28.6% anti-lambda serum and precipitated 100% anti-kappa serum; that more sites responsive to the anti-kappa

antibody are present after alkali-treatment, but that precipitating power with anti-lambda serum is retained is in line with the thought that carrageenan exists as a family of molecules of a number of forms. The intermediate fraction may represent a transitional stage in the biosynthesis of the kappa fraction from the lambda fraction (Rees, 1965). On account of the contamination of the immunizing agent, (lambda preparation with 3,6-anhydrogalactose) the presence of some anti-kappa carrageenan antibody is possible. That would account probably for the precipitation of alkali-treated G. acicularis, lambda (17), C. crispus, kappa (23), G. stellata, kappa (29), and G. stellata, lambda (27), by anti-lambda serum.

Terminal groups of protein antigens play a dominant role in antigenic specificity. Studies on various pneumococcal polysaccharides have shown that terminal non-reducing ends are of major importance in sugars (Heidelberger, 1960). Mage and Kabat (1963) found that the linear polysaccharide, SIII from Pneumococcus, decreased in binding energy with oligosaccharides up to the hexasaccharide. Robbins and Uchida (1962) and Uchida, Robbins and Luria (1963) demonstrated that antigenic groups occur in the interior of many polysaccharide chains and that activity of such groups was the result of tertiary structure. These groups had preferential high affinity for the antibody and such groups were termed the immunodominant groups (Luderitz, Staub and Westphal, 1966). This concept is important for heteropolysaccharides as it increases the number of heterogeneous populations of antibody molecules that can be formed to a single antigenic determinant. Kabat (1957) noticed that in the dextran molecule there was free rotation around the α-1,6 glycosyl bond, that a hexasaccharide may exist in several conformations in aqueous solution, and

that antibodies may be formed to all conformations. Carrageenan in solutions of 0.9% saline are believed to exist as random coils (O. Smidsrød, personal communication). Models of kappa carrageenan indicate that a helical structure may be the most desired conformation and that the replacement of a 2,6-galactose-disulphate for one of several of the 3,6-anhydrogalactose molecules would result in a "kinking" of the coil (G. Mueller, Marine Colloids, personal communication). Alkali-treatment may convert the 2,6-galactose-disulphate to 3,6-anhydrogalactose -2-sulphate with the subsequent straightening of the helix. If the antibody were directed against this 2,6-disulphate 2,6-galactose-disulphate induced conformation, alkali-treatment which would alter it would result in a decrease in precipitation of anti-kappa serum. In fact, precipitation is increased. This either means that the presence of 2,6-galactose-disulphate is not involved, or that increased 3,6-anhydrogalactose increases the number of antigenic sites, or that the reaction is partially non-specific. Conclusions as to the degree of specificity of the reactions involved cannot be stated until the solubility of the antigen-antibody complexes is determined. The amount of increase in precipitation of samples 23, 27, 28 in both anti-lambda and anti-kappa serum remains to be seen. The irregular precipitation of whole fragments from C. crispus (10) and G. pistillata (11) in both anti-lambda sera (Figures 10 and 13) may indicate that the polysaccharides exist as a unit and that in solution only a portion of their antigenic sites were available.

The observation that chondroitin-4-sulphate (1,4-O-β-D-glucopyranosyluronic acid-1,3-2-acetamido-2-deoxy-O-B-D-galactopyranosyl-4-sulphate) and agar (Difco), a mixture of β-1,3-D-galactose and α-1, 4-galactose-4-sulphate, failed to precipitate either anti-lambda serum

or anti-kappa serum is not surprising. The presence of different linkage, sugar moiety and groups make these compounds quite different than the carrageenans. Heidelberger (1960) found that kappa and lambda carrageenan from C. crispus gave non-specific precipitation with pneumococcal polysaccharide SIII and SVIII antisera. Both of these polysaccharides share a common determinant, that is, a series of β-1, 4-glucose linkages.

The lack of reaction between the lambda fraction and the kappa fraction with heterologous antisera indicates that there are distinct antigenic groups within each molecular fraction. The correlation between precipitating power and extraction procedure was significant within certain limits. Fractions designated lambda (Marine Colloids) and Fraction 3 (soluble in 1.5M potassium chloride, Trondheim, Norway) were relatively homogeneous with respect to ability to precipitate anti-lambda serum, that is, they contained a high proportion of "lambda groups". Similarly, the samples designated kappa carrageenan (Marine Colloids) and Fraction 1 (insoluble in 0.0625M potassium chloride) were relatively homogeneous with respect to ability to precipitate anti-kappa serum. The intermediate fractions varied somewhat. Material precipitated between 0.0625M and 1.5M potassium chloride for G. pistillata (13) and G. acicularis (15) was predominantly lambda carrageenan; whereas material precipitated from C. crispus (19) and G. stellata (25) was predominantly of the kappa type. Thus, the chemical composition of two fractions of carrageenan from two species precipitated at the same potassium chloride molarity cannot be equated to each other. This conclusion is in accordance with that of Pernas et al. (1967) who found that 3,6-anhydrogalactose and half ester sulphate content varied from species to species at a particular molarity of potassium chloride.

The composition of the antigenic group can only be hinted at; precipitin data seem to indicate that 3,6-anhydrogalactose is involved in the "kappa" specificity. What imparts specificity to the lambda fraction is not known.

The extent to which linkage of units or groups of half ester sulphates and 3,6-anhydrogalactose participate in the specificity of the molecules will remain unknown until inhibition studies with homologous polysaccharides are carried out. The implications of such studies in the determination of a tertiary structure of polysaccharides is a stimulus for further investigation.

SUMMARY

Both kappa and lambda carrageenan, sulphated galactans extracted from Chondrus crispus, elicited the formation of precipitating antibodies (precipitins). The carrageenan - anti - carrageenan system follows the antigen-antibody kinetics of other well-known anti-polysaccharide systems. Both the lambda and kappa fractions of the carrageenans showed a high degree of immunochemical specificity; namely, little reaction was detected between antibody and heterologous antigen. Molecular weight determinations of the kappa carrageenan antigen according to the equation of Boyd and Hooker gave a value of 290,000 which is in agreement with other investigators using physical methods; a value of 350,000 for the lambda carrageenan antigen also is in accordance with other investigations. Samples of carrageenans soluble in 1.5M potassium chloride were immunochemically similar to the immunizing antigen, lambda carrageenan (C. crispus); fractions insoluble in 0.0625M potassium chloride were immunochemically similar to the kappa carrageenan from C. crispus which had been used as an immunizing agent. The fractions insoluble in 0.0625M to 1.5M potassium chloride varied in ability to precipitate either anti-lambda serum or anti-kappa serum; this testifies to their heterogeneity. Use was made of the precipitin reaction of homologous antigens and heterologous antisera to give some indication of the characteristics of the kappa and lambda determinants. 3,6-anhydrogalactose appears to be intimately involved in determining the kappa determinant(s); indications concerning the lambda determinant(s) are not clear.

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