GASTROINTESTINAL TOXICITY OF CARRAGEENAN IN THE NEWBORN GUINEA PIG

by

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ABSTRACT

The carrageenans (C) are a heterogeneous group of sulfated polysaccharides extracted from algae and used extensively by the food industry as a non-nutritive food additive in dairy products (including infant formula). Previous toxicity studies using adult animals have shown that C produced ulcers in the gastrointestinal (GI) tract only after prolonged (20 day), high level exposure.

The toxicity of oral C in newborn guinea pigs (GP) was examined. GP litter mates were gavage fed (3x daily) with 5 cc of either 1% "food grade" C (N=12). 1% lambda C (N=12), distilled water (N=7), or left untreated (N=6). All GP were separated from their mothers during the treatment. After either 7 doses (3 days) or 12 doses (5 days), blood (3ml) was withdrawn by cardiac puncture, the GP were anesthetized and the GI tract excised. Sections of GI tract (2-3 mm) were either fixed in formalin or frozen in isopentane. Histopathological examination revealed that 3-day treatments of lambda C produced an increase in cecal ulceration, i.e., necrosis and hemorrhage, over that of controls (p less than 0.05). Although specific fluorescent antibody studies revealed positive staining in treated GP GI tissues it remains to be determined whether the toxic effect of lambda C in newborn GP is due to a topical effect alone or whether it is absorbed through the intestinal mucosa.

INTRODUCTION

Carrageenan is the generic name for a heterogeneous group of sulfated polygalactans which can be isolated from alga including many varieties of red seaweed. The alga Chondrus Crispus, known as carrageenan or "Irish moss", is the principal commercial source of carrageenan.

Carrageenan forms a gel in solution and by increasing the viscosity of milk products is able to suspend the proteins (casein). The food industry uses "food grade" carrageenan extensively to prevent whey separation and ice crystal formation in ice cream, to stabilize whipped products and to prevent denatured protein molecules from coalescing and forming a protein precipitate in milk products (e.g., pre-mixed infant formulas). The absorption and toxicity of orally administered carrageenan has not been completely described, and the extensive use of this nonessential food additive explains the interest in it.

Chemistry of Carrageenan

Native or "food grade" carrageenan can be fractionated into two separate copolymers by potassium chloride. One fraction, which gels in the presence of potassium ion, was designated kappa (κ) carrageenan. The other fraction which is insensitive to potassium ion, was named lambda (λ) carrageenan. Kappa and lambda carrageenan represent, respectively, approximately 60% and 40% of an unfractionated extract (DiRosa, 1972).

Kappa carrageenan (Figure 1) is composed of approximately equimolar amounts of sulfated D-galactose and 3,6-anhydro-D-galactose residues. It has a branched structure with a molecular weight of 1.8 to 3.2×10^5 (Johnston and McCandless, 1968). Lambda carrageenan (Figure 2) is composed almost entirely of sulfated D-galactose with a molecular weight od 3.5×10^5 (Johnston and McCandless, 1968). These carrageenan copolymers differ on the basis of molecular weight, cross-linking within the disaccharide monomer, and the sites and degrees of sulfation. However, they both possess the same alternating α (1-3) and β (1-4) linkages.

Mild acid hydrolysis of native carrageenan reduces molecular size while maintaining sulfate content (Black et al., 1965). This produces a degraded carrageenan with a molecular weight less than 30,000 which has often been used for characterization studies because the high viscosity of native (undegraded) carrageenan makes the use of anything other than dilute solutions difficult or impossible.

Although _Chondrus cripus_ is the principal source of food grade carrageenan, copolymers with similar composition and physical properties have been isolated from several other seaweeds, in particular _Gigartina stellata_. In addition, a third carrageenan polymer designated the iota (ι) form has been extracted from the alga _Eucheuma Spinosum_ and _Eucheuma Cottonii_.

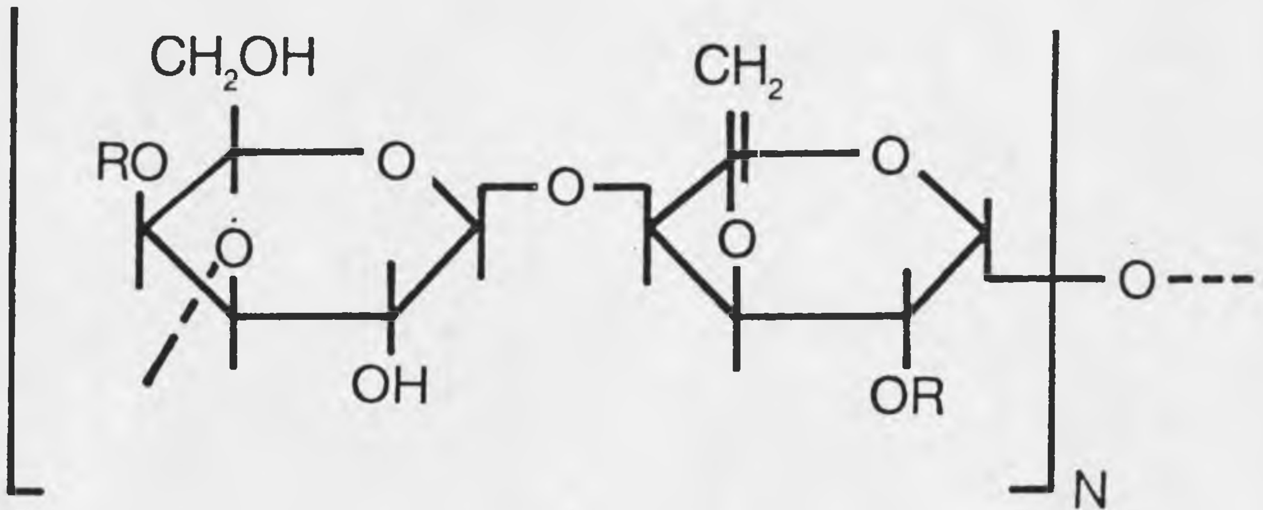

Figure 1. Kappa Carrageenan Structure.

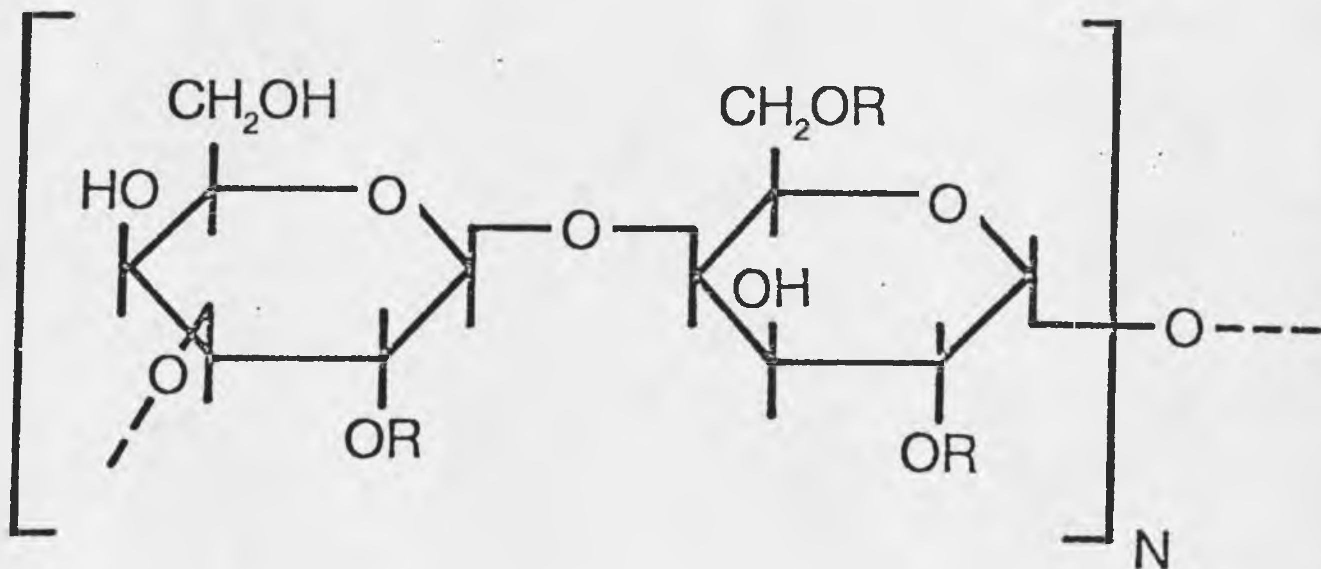

Figure 2. Lambda Carrageenan Structure.

Biological Activity of Carrageenan

The biological actions of the carrageenans can be explained by their chemical configurations. They are large, strongly electronegative molecules that are capable of ionic binding to proteins resulting in competitive nonspecific inhibition of enzymes or co-factors.

Carrageenan is antigenic although it is not known whether this is an intrinsic property or if it becomes antigenic after adsorption to some tissue or some body protein. Serial injections of lambda and kappa carrageenan (25 mg) into rabbits resulted in the formation of precipitins (Johnston and McCandless, 1972). Carrageenan prevents the fixation of C'1 or interferes with a component of complement that precedes the fixation of C'1. This anticomplement activity occurs both in vivo and in vitro (Davies, 1963, 1965). It also appears to inactivate both thrombin (Anderson and Duncan, 1965) and Hageman Factor (Schwartz and Kellermeyer, 1969). Carrageenan therefore produces a dual dose-dependent effect on clotting time (anti-coagulant at higher doses [3 mg/kg] and pro-coagulant at lower ones [1 mg/kg].

A variety of carrageenans of differing molecular weights have been introduced into a variety of mature laboratory animals in varying doses and routes of administration. Intravenous administration of undegraded carrageenan (50 mg) to rabbits resulted in death within 48 hours. Autopsy revealed diffuse renal cortical necrosis and widespread capillary thromboses (Anderson, 1967). Subcutaneous injection in the abdominal wall of the guinea pig produces a hemorrhagic granuloma rich in collagen. This granuloma is in a highly active metabolic state and

is eventually replaced by adipose tissue (Williams, 1957). In this model the intracellular localization of lambda (but not kappa) carrageenan in the macrophages of the granuloma has been demonstrated using fluorescent antibodies (Richer and McCandless, 1972).

Carrageenan is used to induce edema formation in the rat foot. Swelling is elicited by the subplantar injection of 0.05 ml of a 1% carrageenan suspension in saline. This inflammatory response is thought to be brought about by local activation of the complement system (DiRosa, 1972). This model is recognized as an acceptable screening test for antiflammatory agents, and has been used to assay the anti-inflammatory activities of drugs including aspirin, butazolidin, mefanamic acid and indomethacin.

Structure/activity relationships have not been conclusively proven in the various carrageenans, but some facts are evident. Lambda carrageenan, which has 50% more unsubstituted C_6 groups than Kappa carrageenan, is more active as an irritant in eliciting acute (Atkinson, 1962) or chronic inflammatory responses (McCandless, 1965). Lambda carrageenan is also more acutely toxic than Kappa when injected intravenously (Anderson and Duncan, 1965).

It is generally accepted that all carrageenans have some toxicity if they are absorbed by or injected into a mammalian system. The use of carrageenan in food products is based on the assumption that the type and amount of carrageenan used is neither absorbed nor toxic to man. The safety of carrageenan was first questioned when Watt and Marcus (1969) described an 80% occurrence of cecal and colonic

ulceration in guinea pigs, rats, mice and rabbits fed 5% degraded (molecular weight less than 40,000) or 1% undegraded (molecular weight greater than 100,000) carrageenan in drinking water for 30 days. Grasso (1973) also produced ulcers in guinea pigs (but not in rats and hamsters) with these same degraded carrageenans.

In 1972 the Food and Drug Administration reviewed the toxicity of carrageenan (including studies of their own) and concluded that the native carrageenan used in food, at its present and anticipated level of use, did not constitute a hazard, but that degraded carrageenan was toxic. To insure against the introduction of degraded carrageenan into the food supply, they stipulated that carrageenan have an average molecular weight greater than 100,000. The joint FDA-WHO Expert Committee on Food Additives (World Health Organization, 1970) established an acceptable daily intake for man of 500 mg/kg body weight/day. These recommendations were based on a variety of animal toxicity studies, not all of which were negative.

Ensgster and Abraham (1976) reported a 100% occurrence of cecal ulceration in adult guinea pigs fed 5% degraded carrageenan in the drinking water for 14 days, but observed no lesions when adult guinea pigs were fed either 1% undegraded kappa or lambda carrageenan. Pitman, Goldberg, and Coulstor (1976) claimed that only degraded carrageenan is absorbed, but these authors also stated that high molecular weight carrageenans could be degraded during passage through the gastrointestinal tract. They also stated that the amount of carrageenan absorbed was species dependent.

Since the permeability of the gastrointestinal tract to many substances is a function of both maturity and species (Walker and Isselbacher, 1974), it is possible that the absorption (and toxicity) of carrageenan is also age related.

There is confusion in the literature concerning certain effects of carrageenan ("food grade" unless otherwise stated) on biological systems. In addition, comprehensive studies on the effects of carrageenan on the intestinal morphology of neonatal animals have not been described.

Carrageenan is toxic to living tissue when it is present inter- or intra-cellularly. The absence of human toxicity from ingested carrageenan appears to be due to its nonabsorption by the mature gastrointestinal tract. This non-absorption is apparently the result of the high molecular weight of undegraded carrageenan, which is too large to penetrate the epithelial barrier presented by the mature gastrointestinal mucosa. However, this lack of absorption in the adult does not guarantee the same lack in the neonate. It is known, for example, that the immature gastrointestinal tract is permeable to gamma globulins (M.W. 1.5×10^5) and other macromolecules (Walker and Isselbacher, 1974; Goldstein, Anderson, and Brown, 1979) despite the lack of specific receptor sites for macromolecular attachment to cellular membranes in neonates. These facts are consistent with a non-selective uptake of macromolecules by a pinocytotic mechanism occurring throughout the intestine (Walker and Isselbacher, 1974). The absorption of compounds by such a mechanism is a function only of the concentration of macromolecules at the mucosal surface.

The absorption of antigenic macromolecules by the immature gastrointestinal tract is related to the degree of local immunity. Secretory immunoglobulin A (SIgA) inhibits the absorption of antigens by decreasing their adherence to intestinal surfaces. Maternal colostrum is the only source of SIgA and passive local immunity. Selner, Merrill, and Claman (1968) have shown that very little active local immunity develops in the first two weeks postpartum. The absorption of antigens such as carrageenan may be enhanced in those premature or full term infants who are exposed to carrageenan containing formulas without the protective presence of SIgA.

It is also possible that the stress, medications, environment, circulatory changes, respiratory problems and/or metabolic problems of an infant in the nursery further alter the permeability of the immature gastrointestinal tract; thus, the potential for carrageenan absorption and toxicity in the neonate exists. Such toxicity could either cause significant gastrointestinal damage on its own or exacerbate gastro-intestinal damage already present.

This toxic potential is especially important for two reasons. First, carrageenan is used purely for the convenience of using pre-mixed suspended formula. It has no nutritional value. Powdered infant formulas without carrageenan are available but require suspension and hand mixing prior to use. Premixed noncarrageenan-containing formulas could be made although they would separate on standing as does mothers' milk. This would be less appealing visually, but equally nutritious. Second, there is a possible relationship between carrageenan toxicity and a syndrome called necrotizing enterocolitis (NEC).

Numerous clinical disease states, particularly infectious diseases, may be initiated in the neonate by inadequate intestinal integrity which leads to penetration of the intestinal mucosal barrier by bacteria and antigens. NEC is a highly lethal disease occurring most commonly in premature low birth weight infants (Santulli et al., 1975). The syndrome is characterized by ischemic necrosis of the bowel wall with rapidly progressing clinical signs of vomiting, gastric retention, bloody stools, abdominal distention, and often intestinal wall invasion by gas forming bacteria, at times leading to perforation. In NEC the integrity of the intestinal mucosal barriers is reduced, thus allowing bacterial invasion. NEC appears to result from a variety of causes including circulatory ischemia, hypoxia or anoxia of the gut wall or direct toxic injury.

It is possible that hyperosmolar formulas or food additives in formula (such as carrageenan) contribute to NEC either by initiating direct mucosal damage or by exacerbating non-existing mucosal damage. The latter is especially possible for carrageenan since it has been so consistently shown to be toxic once absorbed. Documentation of NEC in breast fed infants is rare. Santulli et al. (1975) found that 63 out of 64 infants with NEC were fed prior to developing the disease; Kliegman et al. (1979) and Moriarty et al. (1979) stated that most infants were fed pre-mixed formula prior to the onset of NEC. These facts are consistent with either a toxic factor(s) in formula or the lack of some protective factor(s) in formula which is (are) present in breast milk.

Purpose of This Investigation

The purpose of this study is to use histopathological techniques to determine whether carrageenan is absorbed by the immature gastrointestinal tract or if it is more toxic to newborn than adult animals.

MATERIALS AND METHODS

The carrageenans used in this study were calcium carrageenan, i.e., "food grade" (Lot #152306, Seakem Brand, Marine Colloids, Inc., Springfield, N. J.), and lambda carrageenan (Lot #87C-0361, Sigma Chemical Company, St. Louis, Mo.)

A 1% solution of carrageenan was prepared by dissolving 2 grams in 200 ml double distilled water at 45° C. The osmolality of the solution was determined using a Westcor Model 5100 Vapor Pressure Osmometer. The osmolalities were: double distilled water 35 mOsm/kg water, 1% calcium carrageenan 68 mOsm/kg water, and 1% lambda carrageenan 48 mOsm/kg water.

The adult guinea pigs (Hartley strain, Southwestern Labs, Tucson, Arizona) used in this study for breeding purposes were fed Purina guinea pig chow and water ad libitum. Studies were conducted using newborn (0-16 hours) guinea pig litter mates. The pups were removed from their mother, weighed and sexed. The selection of litter mates for a treatment regimen was random, with regard to weight (control: 87 grams ± 15; experimental: 88 grams ±11) and sex but runts (<50 grams) were not used. A total of (N=37) animals were studied. Litter mates (2-4 animals/litter) were treated with one of the following: distilled water (N=7), 1% calcium carrageenan (N=12), 1% lambda carrageenan (N=12), or they received no treatment at all (N=6). The pups were gavage fed (5 cc) using a No. 5 French (National Catheter Corporation, Argle, N. J.) infant feeding tube. Feedings

were administered at 0900, 1300, and 1700 hours each day for a total of 7 feedings (2.5 days). A second study was conducted in which twelve feedings (5 days) were administered. All animals were weighed at 0900 and 1700 hours. The animals treated with carrageenan received approximately 1.5 grams/kg/24 hours. At 1700 hours the guinea pigs were placed in the cage with their mother. At this time all solid food was removed from the cage so that breast feeding was the only source of nutrition for the guinea pig pups. Four hours after the last feeding, 2-3 ml whole blood was obtained by cardiac puncture, allowed to coagulate and placed on ice. The serum was collected and frozen (-70° C). After the blood was obtained, each of the pups was anesthetized with ether. At this time, 1-3 ml urine was withdrawn by direct needle aspiration from the animal's bladder and was frozen at -30° C. The entire gastrointestinal tract was removed. Under ether anesthesia, duplicate 2-3 mm sections of stomach, duodenum, ileum, jejunum, cecum and colon were excised. One section of gastrointestinal tract was then fixed in 10% phosphate buffered formalin (furnished by the Division of Animal Resources at the Arizona Health Sciences Center). The adjacent tissue section was then placed in a vial of isopentane (Mallinckrodt Chemical Company) which had been pre-cooled in liquid nitrogen. The vial containing the tissue was then returned to the liquid nitrogen to be snap frozen and was then stored in a freezer (-70° C).

The formalin fixed tissues were subsequently stained with hematoxylin and eosin (H and E) and toluidine blue. All histopathology was performed by Susan E. Wilson, D.V.M., M.S., who was unaware of the treatment that each animal had received.

The chi-square, which measures the goodness of fit between two frequencies, was used in the statistical analysis of the histopathological data. The chi-square formula is:

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)}{E_i}$$

where O_i is the observed frequency and E_i is the expected frequency. A p value of 0.10 was used as an indicator of statistical significance.

Urine Analysis

The methods of Beattie et al. (1970) were adapted for the qualitative spectrophotometric determination of urinary metachromasia. The urine specimens (0.5 ml) were acidified to pH 4-5 using 2 drops concentrated glacial acetic acid. The samples were then diluted with 4.5 ml distilled water and 0.01% toluidine blue solution was added. The mixture was then vortexed briefly. Absorbance was measured against a water blank at 630 nm in a Beckman Spectrophotometer using 1 cm cells. A control, in which water (0.5 ml) replaced the urine, gave an absorbance of 0.65 ± .01. An absorbance of less than 0.65 indicated the presence of carrageenan. A quantitative estimate of carrageenan in urine was also carried out using standards of 0, 5, 10 and 20 mcg/ml (See Table 1 and Figure 3).

Table 1. Quantitative estimate of carrageenan in urine.

Carrageenan Stds.	OD_{630}	Experimental	OD_{630}
Blank	.655	#1	.682
5 mcg/ml	.639	2	.685
10 mcg/ml	.559	3	.680
20 mcg/ml	.515	4	.673
		5	.663
Control	OD_{630}	6	.659
		7	.674
#1	.669	8	.683
2	.674	9	.682
3	.684	10	.666
4	.713	11	.673
5	.655	12	.688
6	.670	13	.659
7	.658	14	.700
8	.780	15	.680
9	.675	16	.669
10	.669	17	.713
$\bar{x}$ = .685		18	.684
		19	.667
		20	.683
		$\bar{x}$ = .678	

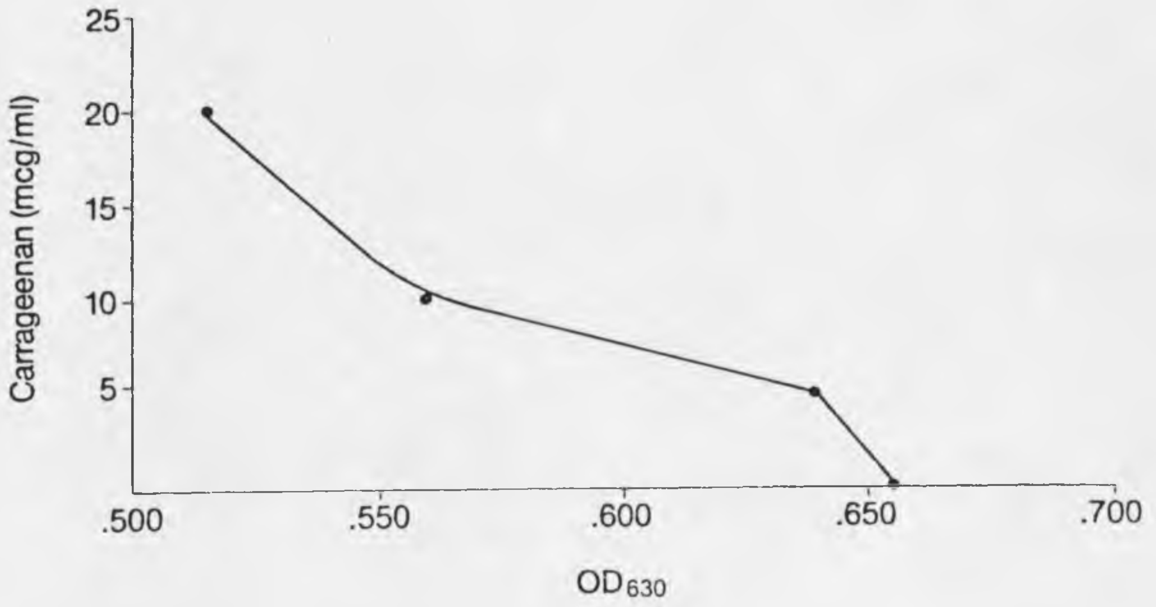

Figure 3. Carrageenan Standard Curve.

Fluorescent Antibody Studies

Specific antibody to λ-Carrageenan was prepared in rabbits using the procedure of Johnston and McCandless (1968). This antibody and a fluorescein conjugated IgG fraction goat-anti rabbit IgG, heavy and light chains, were prepared by Cappel Labs, Cochranville, Pa.

Initially, frozen tissues used for the fluorescent antibody studies were stained using the indirect fluorescent antibody staining technique. Tissue cross sections (5 μm thick) were dehydrated and fixed in fresh acetone for five mintues. The sections were allowed to air dry and then washed twice with phosphate buffered saline. Phosphate buffered saline (PBS) was prepared by combining 84 ml 0.2 M NaH_2PO_4 (27.6 Gm/L), 16 ml 0.2 M Na_2HPO_4 (28.4 Gm/L), 17 Gm NaCl, and diluting this mixture to 2 liters. This .01 M PO_4 buffered .15 M NaCl solution was then brought to pH 7.35 using 4 N NaOH. After the slides containing the tissue sections were placed in a container with a damp towel, a drop of specific carrageenan antibody was placed on the tissue section and the container was covered. The tissue sections were incubated at 27° C for 45 minutes. The sections were then washed again with PBS. A drop of fluorescein conjugated anti-rabbit IgG was then placed on the tissue sections and incubated in the same covered container at 27° C for 45 minutes. After washing with PBS and wiping off excess fluid from the slide, the sections were mounted under a coverslip in a non fluorescing medium of 50% glycerol in PBS. Tissue sections were then stored at -70° C until they were examined under a fluorescent microscope. Tissues were obtained from both treatment groups, i.e., experimental and control.

A positive tissue control was obtained using the technique of Richer and McCandless (1968). Five cc of a 1% carrageenan solution (λ) was injected subcutaneously into the abdomen of a mature guinea pig. The granuloma produced by the carrageenan was excised after seven days. Small blocks of tissue were sectionad and stored at -70° C. A negative control was obtained by injecting another guinea pig similarly with distilled water and taking a tissue section after seven days.

Preparation of FITC-Tagged Gamma Globulin

In addition to using the indirect fluorescent antibody staining technique, a fluorescein isothiocyanate (FITC) labelled gamma globulin was prepared using the specific arrrageenan antibody prepared by Cappel Labs. The following procedure was used to quantitate, isolate, label and purify this antibody.

The Cappel antiserum suspension (40 ml) was mixed with 40 ml 0.01 M phosphate buffer (pH adjusted to 7.0 with 4 N NaOH). While stirring this mixture, 80 ml saturated ammonium sulfate solution was added dropwise. The solution was stirred overnight at 4°C and then centrifuged at 7,000 rpm for 20 minutes at 4° C. After discarding the supernatant, the precipitate was washed twice with half saturated ammonium sulfate solution. The precipitate was resuspended in 40 ml 0.01 M phosphate buffer and dialyzed overnight in one liter of 0.01 M phosphate buffer at 4° C.

A protein determination was performed on the suspension using the following formula: OD_{280} x .667 (extinction coefficient of

tyrosine) x dilution = mg/ml protein; mg/ml x volume = mg total protein. The UV absorption of the suspension was measured at 280 nm in a Beckman Spectrophotometer using 1 cm cells with 0.02 N NaOH used as a blank. The total protein yield was 360.5 mg.

A DEAE Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) column was prepared to separate protein fragments from intact gamma globulin. The column volume was approximately 1 ml DEAE/10 mg protein. The column was flushed with 1.0 M phosphate buffer (pH 7.0) in order to charge the resin with phosphate ion. The column was then flushed and equilibrated with 0.01 M PO_4 buffer. The protein was loaded onto the column and eluted with PO_4 buffer. Six ml fractions were collected. The OD_{280} of each sample was read using a PO_4 buffer blank and all samples with absorbances between 0.1-0.3 were pooled (see Figure IV). This procedure yielded 65 mg purified protein from the original 360.5 mg. The pooled samples were pervaporated in dialysis tubing to obtain a protein concentration of 10 mg/ml. The solution was then dialyzed for 24 hours in PBS (1 liter) at 4° C.

FITC was weighed out (0.020 mg FITC/mg protein) and dissolved in a volume of freshly prepared carbonate buffer equal to the volume of protein containing phosphate buffer. The carbonate buffer was made by mixing 100 ml 0.5 M $NaHCO_3$ (4.2 gm/100 ml) and 20 ml 0.5 M Na_2CO_3 (5.3 gm/100 ml). The pH of the solution was brought to 9.2 using 4 N NaOH. The FITC solution was added to the protein solution dropwise while stirring and was left stirring overnight at 4° C. The mixture was dialyzed for 24 hours in 0.01 M PO_4 buffer in 0.05 M NaCl (1 liter)

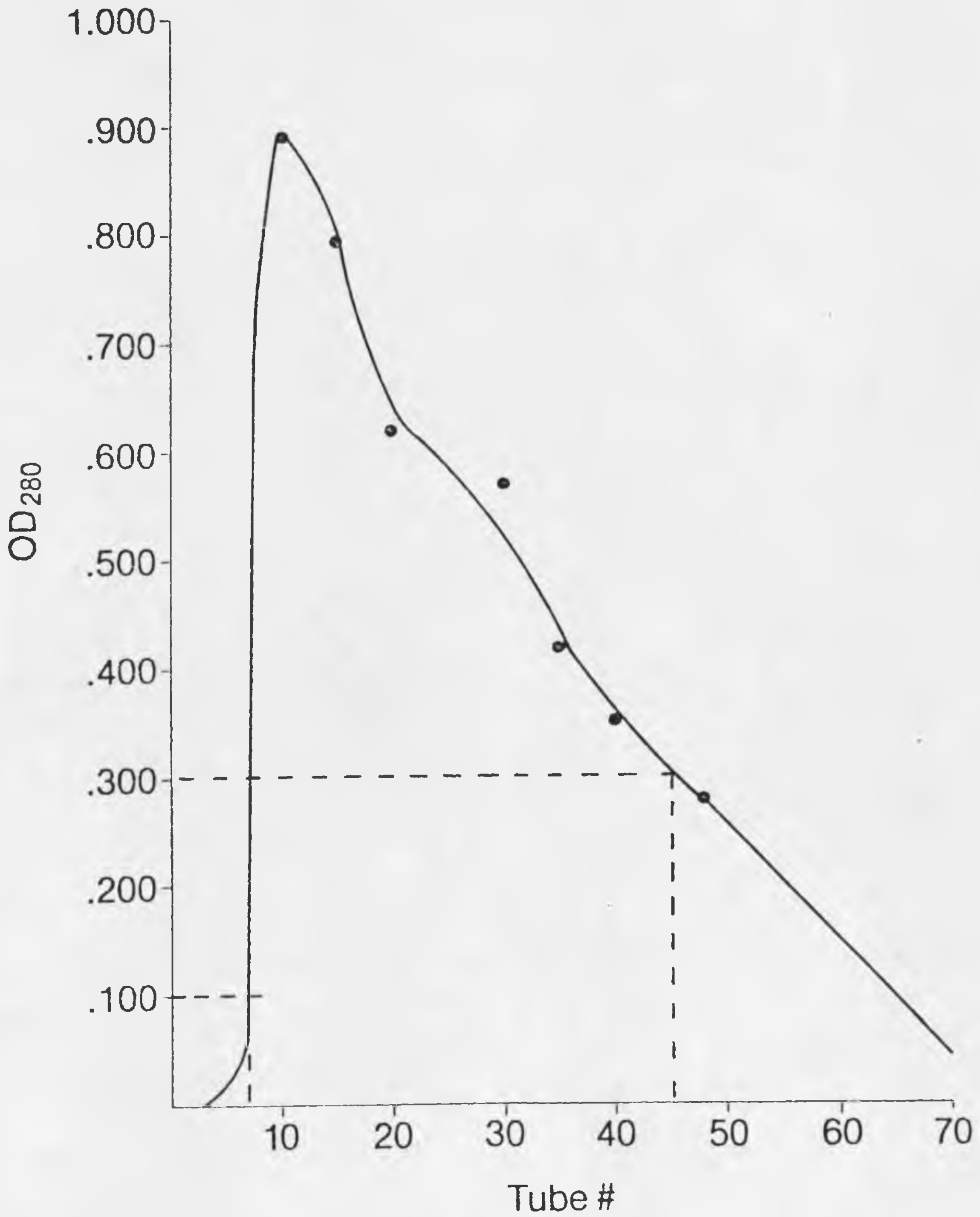

Figure 4. Purified γ-Globulin Pool.

with one change of buffer. The mixture was then applied to the same DEAE Sephacel column which had been used to isolate the gamma globulin. The column was equilibrated with 0.01 M PO_4 buffer in 0.05 M NaCl. A stepwise NaCl gradient was used to elute three major fractions off the column. The three eluents used were 0.05 M, 0.10 M, and 0.30 M NaCl in 0.01 M PO_4 buffer. Once the OD_{280} of a major fraction descended below 0.300, the next strength eluent was added to the column (see Figure V). All fractions having OD_{280} less than 0.100 were discarded.

In order to determine the extent of antibody labelling, a fluorescein/protein labelling ratio was obtained according to the methods of Johnson, Holoborow and Dorling (1978). The OD_{495} (corresponding to the peak absorbance of FITC conjugated to protein) and OD_{280} of the conjugate solution are compared. A dilution of 1:40 of the neat conjugate should have a ratio of unity. Values below 0.5 indicate low labelling and values above 1.5 indicate excessive labelling which may cause nonspecific staining. The highest ratio obtained was determined to be less than 0.5. There was apparent reason for this low labelling.

Antibody Dilutions

In order to obtain the maximal amount of specific antibody tissue labelling with a minimum of nonspecific background staining, several dilutions of antibodies were made and the guinea pig granuloma tissues were stained with them. Table 2 contains the antibody dilution scheme which was used for both the direct and indirect staining techniques.

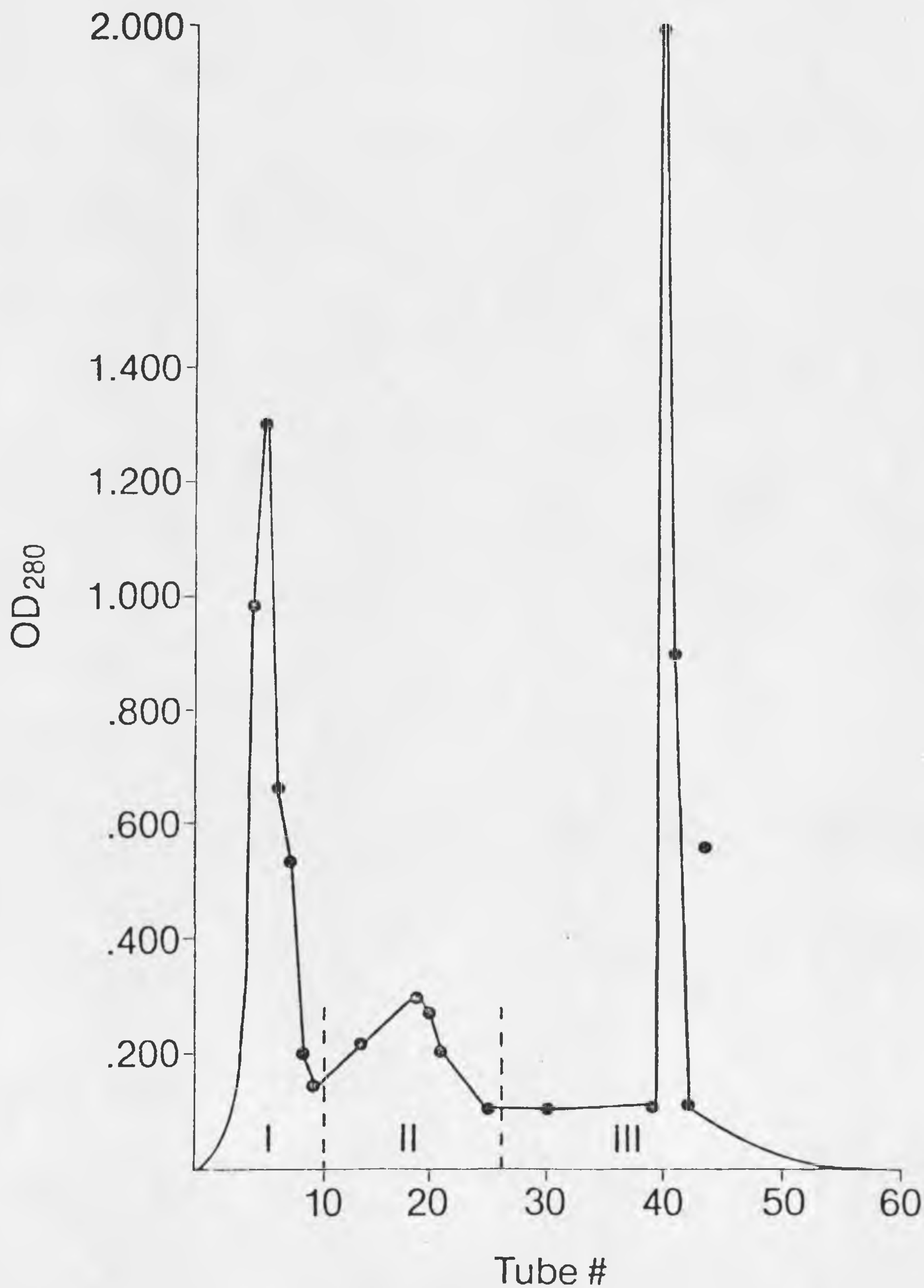

Figure 5. Eluted γ-Globulin/FITC Fractions.

Table 2. Antibody Dilutions.

Rabbit Anti-Carrageenan	Goat Anti-Rabbit IgG
1:5	1:5
1:10	1:5
1:20	1:5
1:40	1:5
1:80	1:5
1:160	1:5
1:5 ← 1:160	1:10
1:5 ← 1:160	1:20
1:5 ← 1:160	1:40
CONTROL { 1:10	PBS
PBS	1:10

Histochemical Studies

Two female mice (C57 Black), mean weight 25 g, were injected with 0.3% lambda carrageenan (0.3 ml) in 0.9% saline to obtain another positive staining tissue control (Steele and Lowes, 1979). The two mice were injected with 0.9 mg via the lateral tail vein. A third mouse was injected with 0.3 ml 0.9% saline as a control. After six hours the mice were sacrificed. Duplicate sections of liver, spleen, kidney, and lungs were taken. One section of each organ was fixed in 10% phosphate buffered formalin and the other was snap frozen in isopentane cooled in liquid nitrogen. Frozen sections were cut and stained with fluorescent antibody specific for lambda carrageenan. Paraffin sections were cut and stained using hematoxylin and eosin, and toluidine blue. The latter is an excellent nonspecific stain for carrageenans, which appears as a brightly purple metachromatic material against a pale blue background (Williams, 1957).

Guinea pig granuloma tissue (positive control) and tissue sections taken from the animals receiving gavage feedings of carrageenan or water were also stained with toluidine blue.

Complement Studies

Guinea pig blood (2-3 ml) obtained by cardiac puncture was allowed to coagulate and the serum was frozen (-70° C). The samples (N=25) were packed in dry ice and shipped by air to Dr. Robert Strunk at National Jewish Hospital in Denver, Co. The samples were assayed for complement (C_1 and CH_{50}).

RESULTS

Spectrophotometric Urine Assay

The urine of carrageenan treated (N=20) and water treated (N=10) guinea pigs was assayed for the presence of carrageenan using a nonspecific qualitative spectrophotometric assay for urinary metachromasia developed by Beattie et al. (1970).

Absorbance was measured against a water blank at 630 nm in a Beckman Spectrophotometer using 1 cm cells. A control, in which water replaced urine, gave an absorbance of 0.65 ± .01. Carrageenan reduces the absorbance of toluidine blue. Absorbance of less than 0.64 indicated the presence of carrageenan. A quantitative estimate of carrageenan concentration can be carried out by comparison to standards containing 0, 5, 10 and 20 mcg/ml (see Figure III).

None of the guinea pig urine samples tested had an absorbance less than 0.64. Carrageenan was not detected in the urine of any of the carrageenan treated animals.

Guinea Pig Histopathology

Histological changes in the gastrointestinal tract of the guinea pigs were graded by the pathologist according to the following scheme:

0 = No significant lesions observed

+1 = Mild changes such as occasional polymorphonuclear cells (PMN) or occasional individual epithelial cell necrosis

+2 = More numerous clusters of PMN in epithelium or more numerous necrotic cells such as in the lamina propria or villi

+3 = Frank enteritis, typhlitis or colitis with numerous PMN, denuded epithelium, macrophage infiltration or necrosis in glands

+4 = Hemorrhage, multifocal in intestine with mild inflammation

+5 = Massive hemorrhage with necrosis, with or without severe inflammatory changes

Lesions found in the small intestinal sections (duodenum, jejunum and ileum) were scored and these numbers were then averaged for each animal. Only one number was recorded then for each animal in each category: small intestine, cecum and colon (see Tables 3 and 4).

Lesions were found in all treatment groups, i.e., water treated control, absolute control (no treatment) and experimental. There was no significant difference between control groups with regard to the incidence or extent of pathology. For this reason, the control groups were combined when comparing control and experimental histopathology.

The incidence of small intestinal lesions in the experimental groups was not significantly different from the control groups. Although the total incidence of small intestinal lesions appears to be

Table 3. Three day study, 7 feedings.

	0		+1		+2		+3		+4		+5	
	S.I.	Cecum	S.I.	Cecum	S.I.	Cecum	S.I.	Cecum	S.I.	Cecum	S.I.	Cecum
$Control_1$ H_2O N=7	3	4	2	1	2	2	0	0	0	0	0	0
$Control_2$ No Treatment N=6	1	0	4	3	1	2	0	1	0	0	0	0
$Experimental_1$ "Food Grade" Carrageenan N=12	5	2	7	6	0	3	0	0	0	1	0	0
$Experimental_2$ Lambda Carrageenan N=12	2	1	9	3	1	3	0	2	0	1	0	2

Table 4. Five day study, 12 feedings.

	0		+1		+2		+3		+4		+5	
	S.I.	Cecum	S.I.	Cecum	S.I.	Cecum	S.I.	Cecum	S.I.	Cecum	S.I.	Cecum
$Control_1$ H_2O N=9	5	4	3	3	1	2	0	0	0	0	0	0
$Control_2$ No Treatment N=3	0	2	3	1	0	0	0	0	0	0	0	0
$Experimental_2$ Lambda Carrageenan N=9	2	3	6	3	1	3	0	0	0	0	0	0

high, 69% control, 83% experimental, this was due mainly to the +1 classification. These are mild changes which may be expected in healthy animals. The incidence of more severe (+2 or greater) small intestinal lesions was very low in all treatment groups.

Carrageenan treated animals were found to have a 30% increase over controls in the incidence of cecal lesions ($p = .08$). Guinea pigs in the 3 day treatment group fed lambda carrageenan had a 35% increase in the more severe types of lesions (+2→+5) over control animals ($p = .06$). When only the most severe lesions demonstrating frank inflammation without (3+) or with hemorrhage (+4→+5) were examined, the increase in incidence was significantly increased ($p = .05$) over control. Two of the animals treated with lambda carrageenan (N=21) were found to have massive multifocal hemorrhage with necrosis and severe inflammatory changes. No lesions of this type were found in any of the control animals (N=25).

Guinea pigs treated for 5 days with lambda carrageenan did not have any significant increase in the incidence or severity of cecal pathology over control animals. In fact, both the incidence ($p = 0.1$) and severity were less in the 5 day versus the 3 day treatment groups.

Many of the colon tissue samples, both control and experimental, were unfortunately lost by the pathologist. The histopathology of the colon was therefore not included in the results.

The animals treated with "food grade" carrageenan had a total incidence of lesions very similar to the control animals (58% vs. 69%). These lesions were all of the mild or "normal" +1 type.

Fluorescent Antibody Studies

The indirect antibody reaction, i.e. rabbit carrageenan antibody incubated with carrageenan induced guinea pig granuloma tissue followed by fluorescein labelled goat antirabbit IgG, was lacking in specificity. With this method there was no consistent difference between the staining of carrageenan induced granuloma (positive) and negative tissues.

Due to the nonspecific results of the indirect staining technique, a direct staining technique was utilized. A pure preparation of rabbit gamma globulin (anticarrageenan antibody) was reacted with FITC in an attempt to produce fluorescein labelled antibody for direct staining studies. However, the antibody produced was of low yield and underlabelled. This procedure was performed twice and the results were unacceptable both times.

Cappel Labs was contracted to prepare an FITC labelled antibody from the original. Direct staining of guinea pig granuloma tissue using this fluorescent antibody was positive and appeared to be specific. Positive staining was clearly seen at 200x and appeared as discrete, intracellular non-nuclear fluorescence in macrophages with a minimum of background fluorescence.

Three tests were used as evidence of antibody specificity:
1) immunoelectrophoresis (IEP) of antibody with antigen (carrageenan) forming a precipitin line (this work was done by Cappel Labs);
2) incubation of carrageenan induced granuloma tissue with unlabelled carrageenan antibody followed by FITC labelled carrageenan antibody;

3) adsorption (three times) of FITC labelled carrageenan antibody with a 1.0% lambda carrageenan solution. This adsorbed antibody was then incubated with granuloma tissue.

The IEP test was positive; this test is the most sensitive and definitive of the tests for antibody specificity. There was a single precipitin band indicative of antibody specificity. The second test was not definitive because although diminished in intensity the granuloma tissue stained positively. In the final test carrageenan adsorbed the fluorescent antibody completely from solution; there was a total absence of fluorescent staining in the granuloma tissue.

Once it appeared that the antibody specifically stained for carrageenan in tissue, the tissues from the five day treated animals were tested for carrageenan absorption using this antibody. No positive staining was detected in any of the tissues from control animals. However, there were several types of positive fluorescent staining patterns seen in the carrageenan treated guinea pig tissues. Positive staining was seen only in carrageenan treated animals; there was a specific pattern of staining and there was specific localization of staining. This positive staining was in tissues with increases in the pathological findings (+3–+5).

Complement Studies

There was no significant difference between control and experimental complement levels, C_1 and CH_{50} (Figures VI, VII). There was a very large variation of values in both the control and experimental groups.

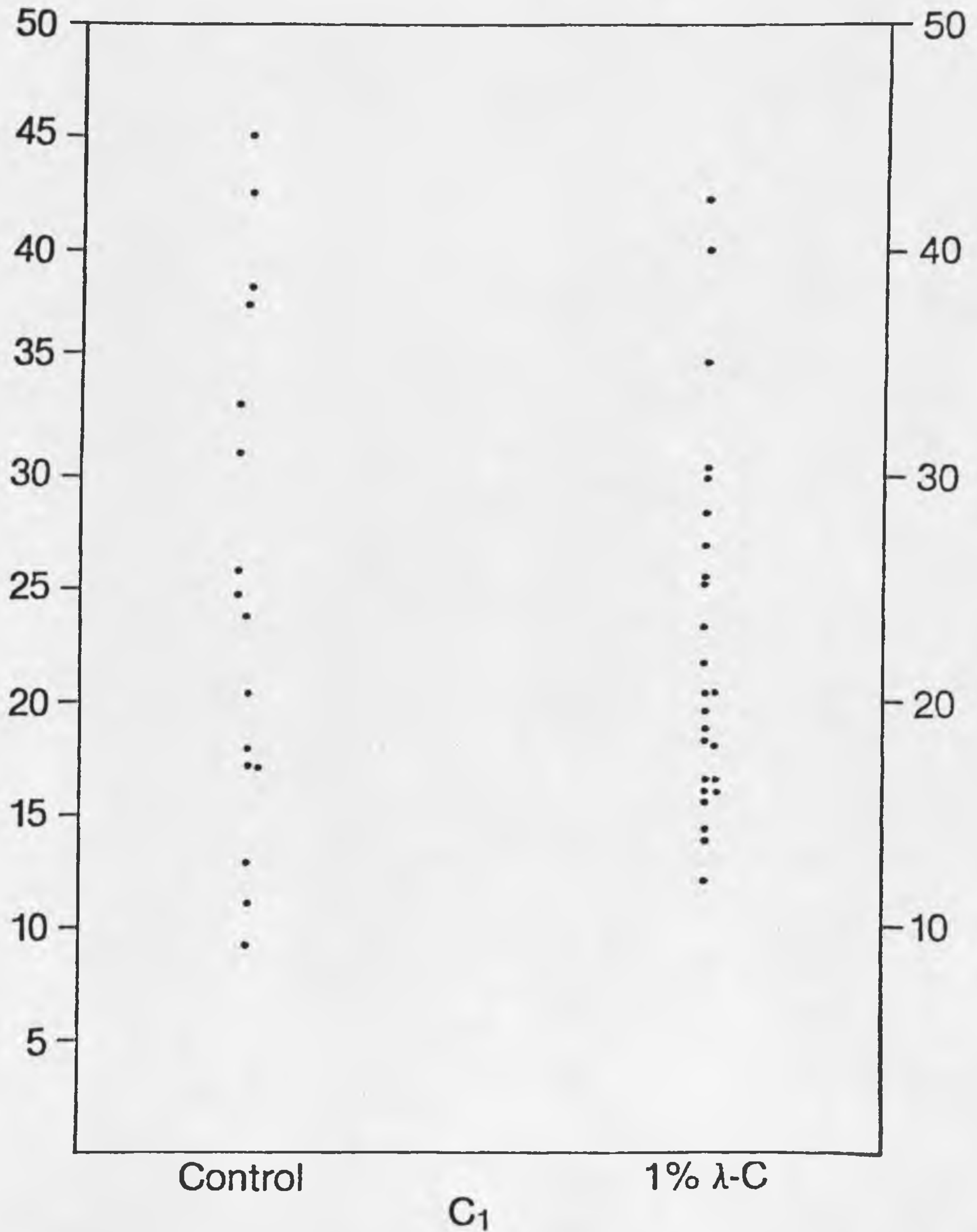

Figure 6. Serum Complement (C_1) Levels.

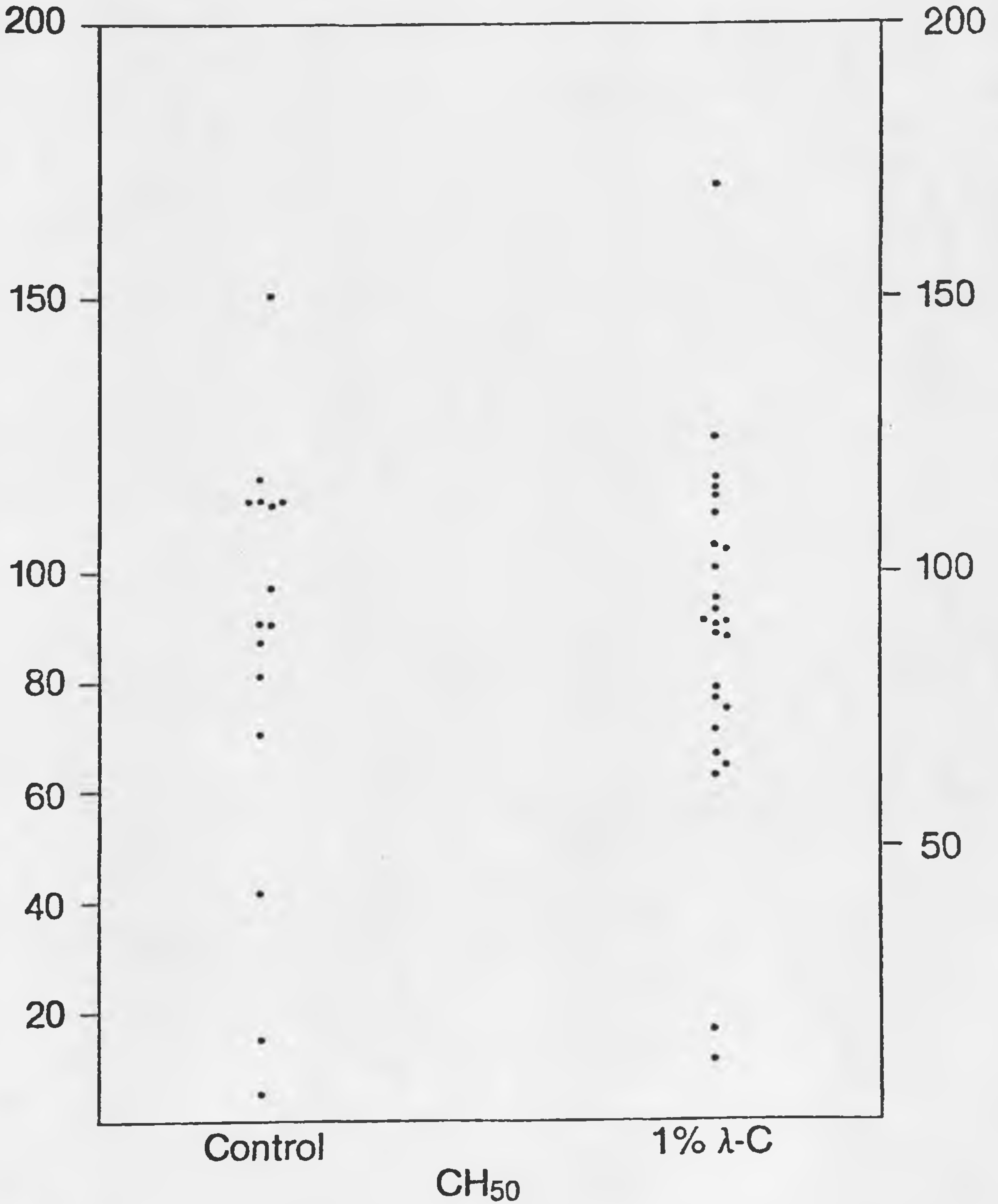

Figure 7. Serum Complement (CH_{50}) Levels.

DISCUSSION

The objectives of this investigation were twofold: to correlate the incidence and severity of guinea pig gastrointestinal histopathology with gavage feedings of carrageenan and to examine these same tissues histochemically for direct evidence of carrageenan deposition and/or absorption.

A histopathological grading system, based on the progressive histological events of inflammation, was devised by the same pathologist who graded the histology slides. Although these evaluations are qualitative and somewhat subjective, the pathologist was unaware of the treatment each animal received.

There is conflicting evidence regarding a dose/response effect. Lambda carrageenan, known to be the more toxic of the "food grade" copolymers, produced an increase in the incidence and severity of lesions in the guinea pig gastrointestinal tract when administered over a three day period, whereas "food grade" carrageenan, which is only thirty five percent lambda, did not. However, if carrageenan produces a direct toxic effect or if it exacerbates preexisting lesions, increased exposure to carrageenan should produce an increase in the incidence or severity of these lesions. Yet in the five day treatment groups there was no demonstrable effect on the gastrointestinal tract. An aspect that must be considered is the higher mortality rate for the animals receiving the five day treatment. Twenty percent (5/26) of the animals in the five day treated group died

as opposed to seven percent (3/40) mortality in the three day group. As the study progressed gavage feedings became increasingly difficult to perform. Some of the animals suffered hypoxic episodes due to laryngospasm following a gavage feeding and some may have died of carrageenan toxicity. Animals in the five day treated group which did develop toxicity may have been selected out because of increased mortality.

In the study maternal colostrum may have exerted a protective effect on the newborn guinea pigs since they were allowed to breast feed every night. This may have been a defect in study design, i.e. allowing the newborns to breast feed rather than eating solid food only, but this more closely approximates the human situation. The extra two days of breast feeding could also contribute to the decrease in histopathology noted in the five day animals.

Two tests were employed to assay for the systemic presence of carrageenan. A spectrophotometric method was employed to assay for carrageenan in the urine. If carrageenan were absorbed it may be degraded metabolically, filtered by the kidney and excreted in the urine. If carrageenan were absorbed into the blood it may have interacted with serum complement. The serum was assayed for C_1 and CH_{50}. Both assays were negative.

The critical question to be answered was whether or not carrageenan crossed the epithelial barrier of the gastrointestinal mucosa. The histochemical stains were chosen to help answer this question. Toluidine blue and fluorescein isothiocyanate labelled

anticarrageenan antibody were used to stain for inter- and intra-cellular carrageenan.

Toluidine blue was too nonspecific. Although it is a bright, sensitive stain for carrageenan, it also stains chondroitin sulfate, other mucopolysaccharides and mast cells. This stain simply produced too many false positives.

False positives were also the major concern of the fluorescent antibody studies. We were unable to bind all of the positive sites (carrageenan) in the carrageenan induced granuloma tissue with unlabelled anticarrageenan antibody in order to make these sites unaccessible to the fluorescent carrageenan antibody. This may have been due to a low affinity or binding capacity of antibody for carrageenan rather than a lack of specificity. Polysaccharides often show a species specific ability to induce antibody formation (Johnston and McCandless, 1972). This raises the question whether the carrageenan become antigenic only after adsorption to some tissue or plasma protein. Perhasp the carrageenan antibody produced in the rabbit has a low affinity or binding capacity for unbound carrageenan in guinea pig tissue because it was not bound to a rabbit tissue protein.

The positive adsoprtion test was not entirely definitive. Carrageenan is known to bind proteins nonspecifically because of its large electronegative charge. Therefore carrageenan may have precipitated out the anticarrageenan antibody in a nonspecific manner.

Because of the uncertainties surrounding the specificity of the antibody and the varied staining patterns seen in the

experimental animals, we are still unsure whether or not carrageenan is absorbed. The possibility remains that carrageenan is deposited on or absorbed by the gastrointestinal mucosa. It is also possible that carrageenan could exert its toxic effect before day five of feeding and then be sloughed or washed away with the necrotic tissue prior to fluorescent antibody staining or cleared by phagocytic cells and carried away.

The decision to use the guinea pig as the test animal in this investigation was based on several immunological and physiological factors. Immunologically, the newborn guinea pig closely resembles the human neonate. Both receive passive immunity transplacentally. This suggests both have a reduced gastrointestinal permeability to macromolecules such as gamma globulin as well as a reduced local immunological response in a neonate deprived of maternal colostrum.

The newborn guinea pigs were required to withstand several highly stressful events. The first stress was separation from the mother. This minimized breast feeding and the protective effect that mother's milk provides. This correlates closely with the human situation in nurseries. The second stress was the gavage feedings of 1% carrageenan, a highly viscous non-nutritive solution. The large volumes used (5 ml) also stressed the animal greatly. Fortunately the guinea pig is unable to vomit. This allowed the animals to be given 1500 mg/kg/day of carrageenan, three times the allowable daily intake established by the FDA-WHO (1972) for human consumption.

But the factors that enabled the guinea pig to withstand our stressful procedures are the same factors that may protect them from

potentially toxic food additives. The guinea pig is a relatively mature animal at birth. It has all its fur, its eyes are open, and it is able to chew and digest solid food at day one.

The physical maturit yof the newborn guinea pig served the animals well. None of the animals included in the data analysis exhibited significant weight losses, physical disabilities, weakness or loss of fur during or as a result of the treatments. There were fifteen animals not included in the data analysis. Excluded animals were: one litter (four animals) which had severe diarrhea, two animals in the three day study (non litter mates) which had severe generalized infections, and two untreated litters (nine animals) in the five day study with a generalized illness which exhibited a high degree of gastrointestinal pathology.

Two drawbacks to this model were the long gestational period and the relatively small litter size. Guinea pig gestation is approximately 70 days and produces a litter of 3-4 pups. Two years were required for a breeding colony of one male and eight females to produce the 68 guinea pigs used in this investigation. This left little time to fully study a second animal model, e.g. newborn rats. Preliminary work with neonatal rats indicates that the LD_{50} of gavage feedings of 1% lambda carrageenan is approximately 300 mg/kg/ day.

CONCLUSIONS

The results of the histopathological and fluorescent antibody studies are not entirely conclusive. The histopathology p value of less than 0.05 does not permit an unequivocal statement regarding the oral toxicity of carrageenan. However, it is evident that lambda carrageenan did exert a deleterious effect on the gastrointestinal tract of the newborn guinea pigs. This effect was noted after only three days of treatment. This is in contrast to previous studies which demonstrated no toxicity in adult guinea pigs until after more than 14 days of exposure.

Fluorescent antibodies specific for lambda carrageenan were localized both in carrageenan induced guinea pig granuloma tissue and gastrointestinal tissues from some treated guinea pigs. Positive staining was not detected in untreated guinea pig gastrointestinal tissues.

The guinea pig may be an appropriate animal model because of its immunologic similarity to the human neonate. However, the guinea pig is also a very mature animal at birth and may not be as susceptible to carrageenan toxicity in a sub-chronic dosing regimen. Another less mature animal (such as the newborn rat) should be treated in a similar dosing regimen to clarify this possibility.

In conclusion, carrageenan, a non-essential potentially toxic food additive, may be absorbed by the newborn guinea pigs; even though

it was not excreted in the urine following oral administration nor did it alter serum complement levels. It remains to be determined whether the toxic effect of carrageenan is due to a topical effect alone or whether it is due to absorption through the intestinal mucosa of the newborn guinea pig. It is also unclear whether absorption occurs in other neonatal animals including humans. These results however reaffirm the need to evaluate the toxicity of this non-essential food additive in newborns.

APPENDIX A

FLUORESCENT ANTIBODY PICTURES

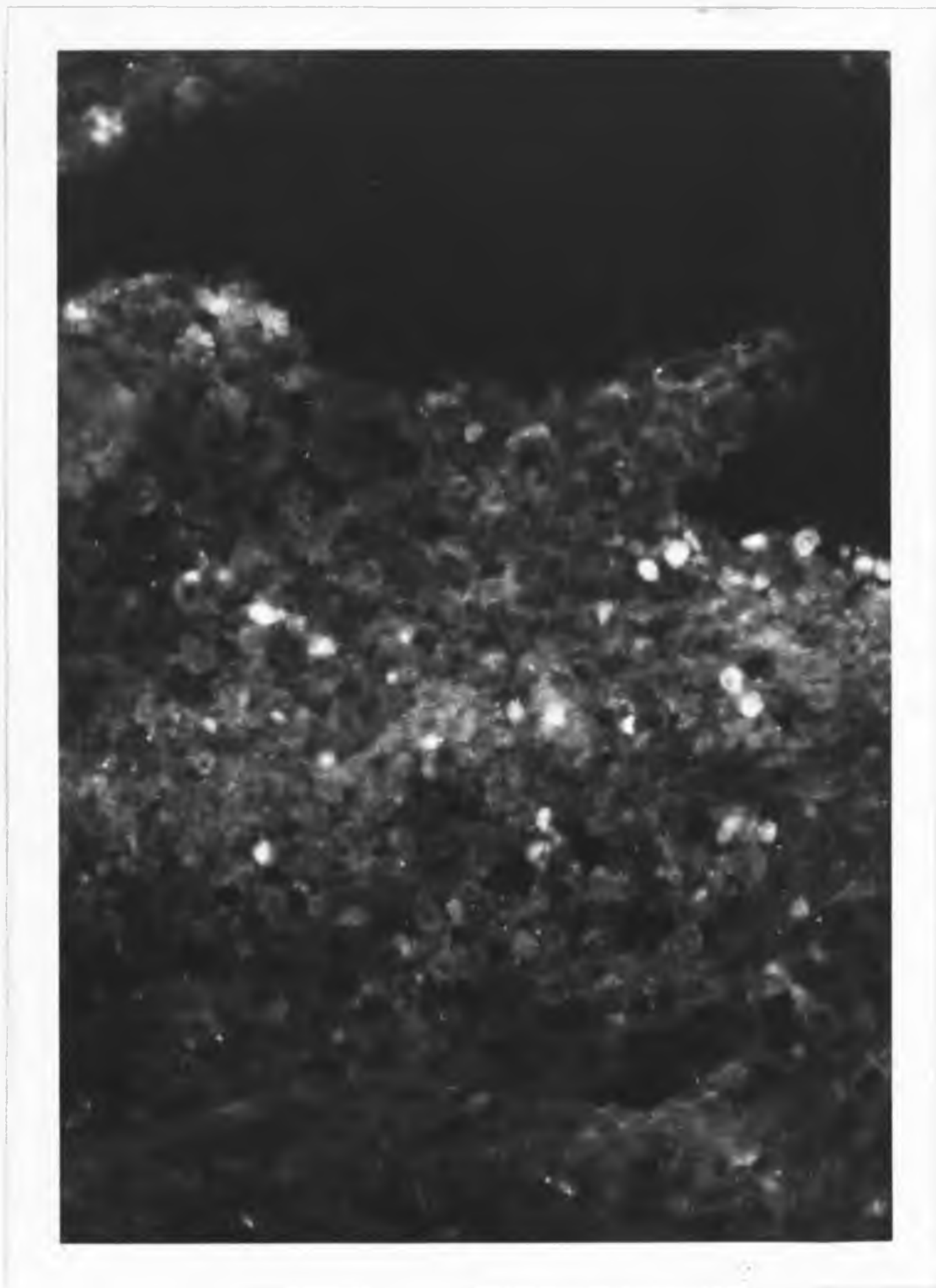

Figure A.1. Positive Fluorescent Antibody Staining of Carrageenan Induced Guinea Pig Granuloma Tissue (200X).

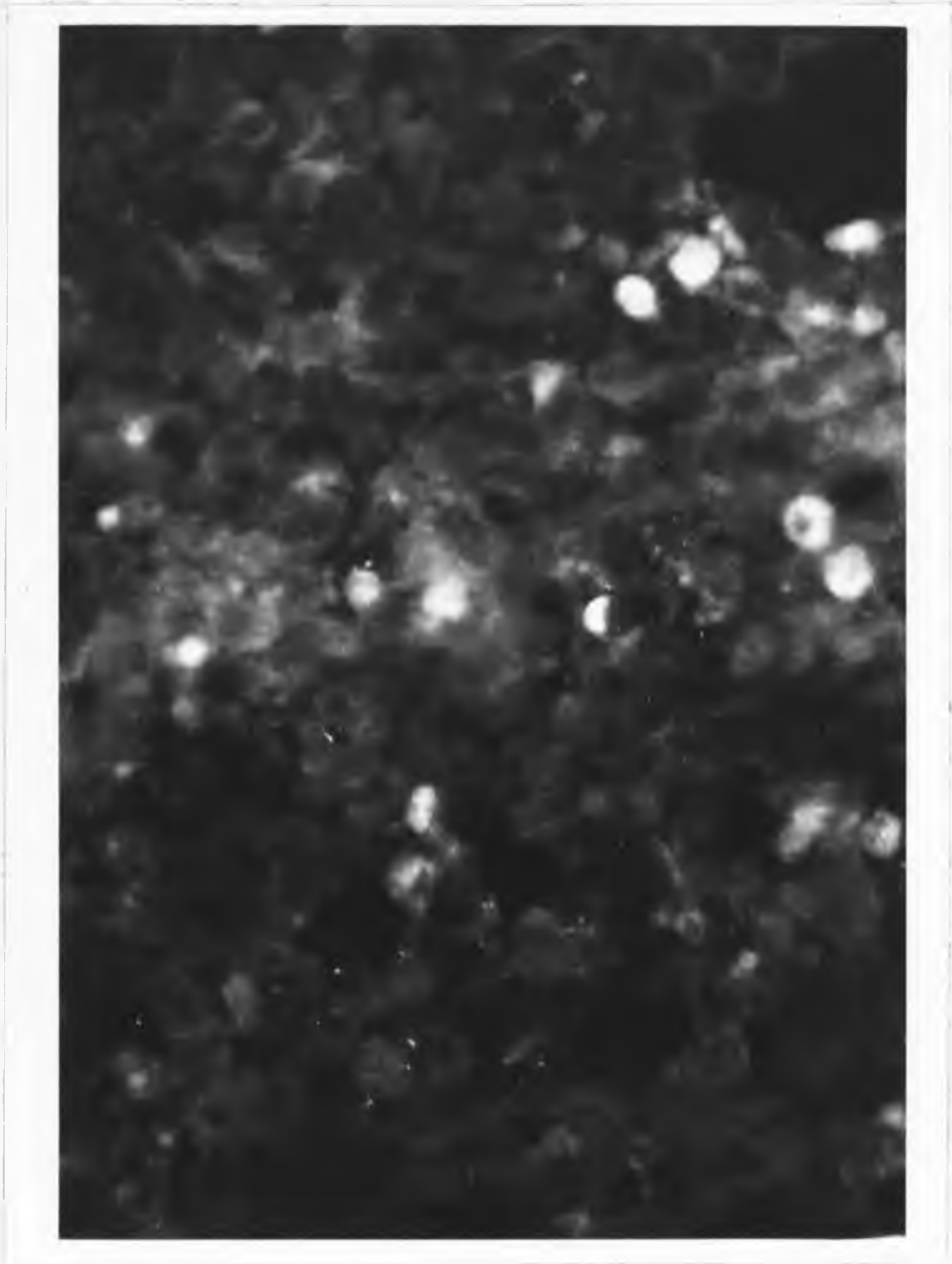

Figure A.2. Positive Fluorescent Antibody Staining of Carrageenan Induced Guinea Pig Granuloma Tissue (400X).

Figure A.3. Granuloma Tissue Incubated with Unlabelled Antibody Then Flourescent Antibody (200X).

Figure A.4. Granuloma Tissue Incubated with Fluorescent Antibody That Had Been Adsorbed by Carrageenan (200X).

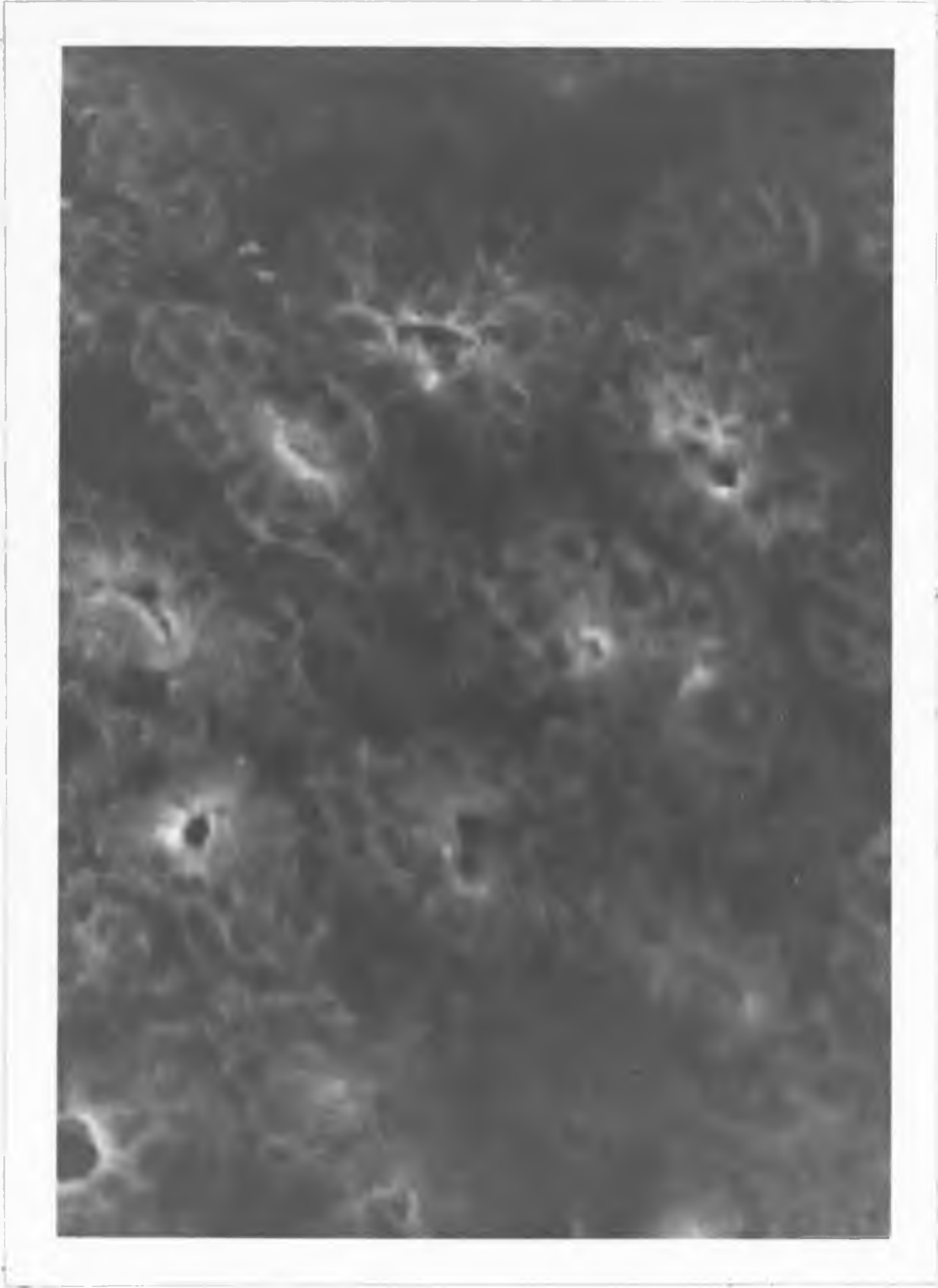

Figure A.5. Untreated Guinea Pig Cecum (200X).

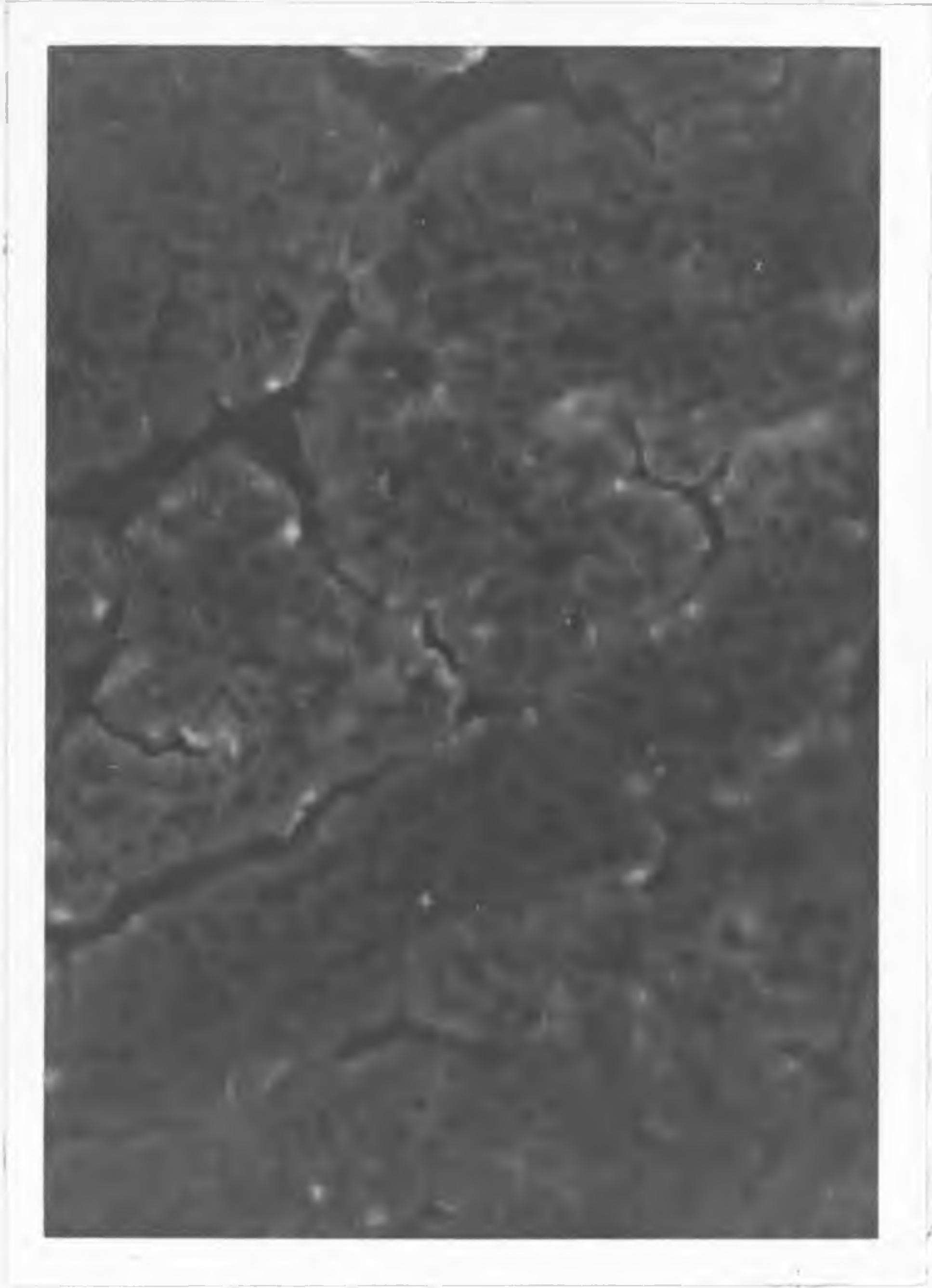

Figure A.6. Water Treated Guinea Pig Cecum (200X).

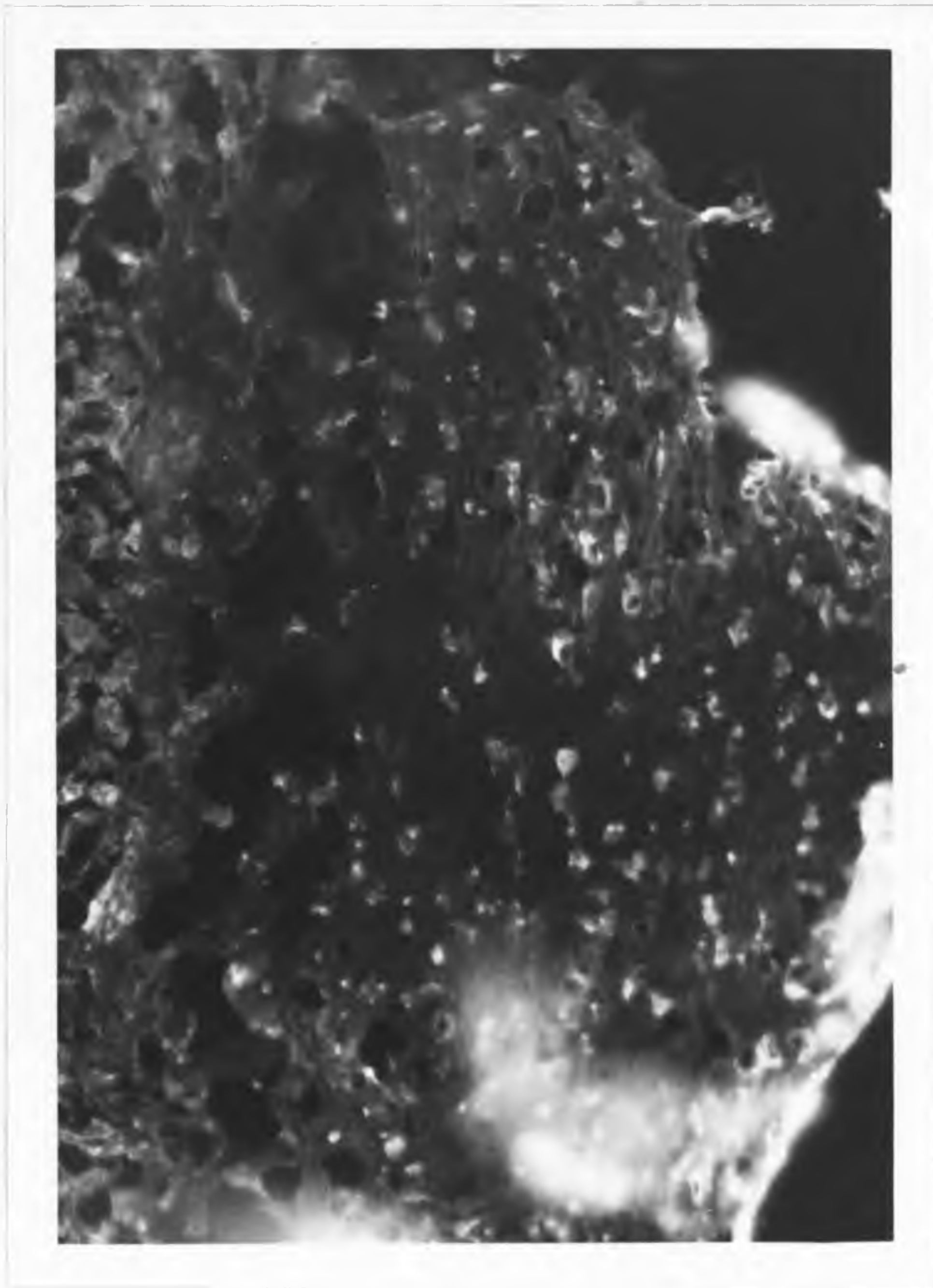

Figure A.7. Lambda Carrageenan Treated Guinea Pig Cecum (200X).

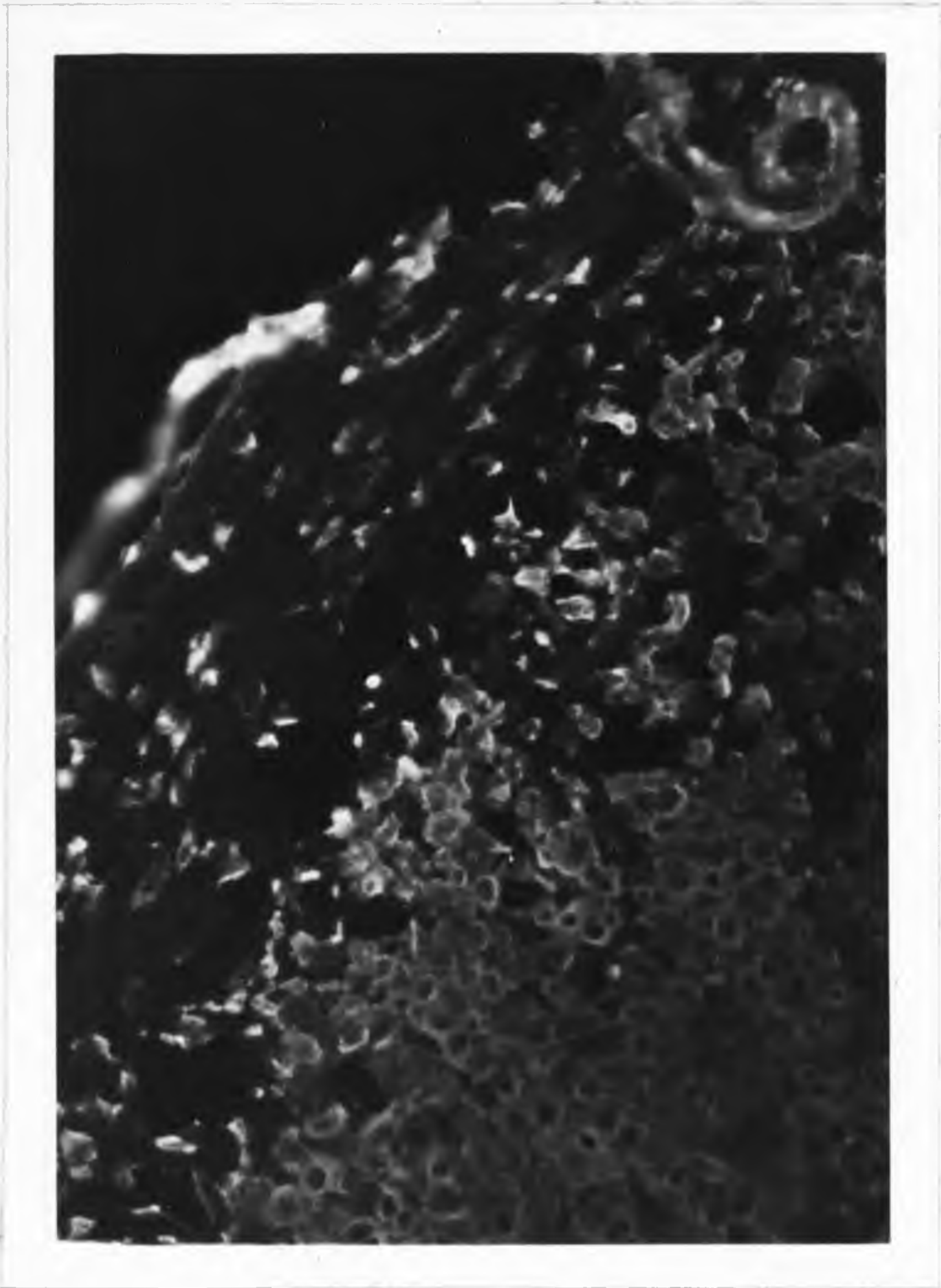

Figure A. 8. Lambda Carrageenan Treated Guinea Pig Cecum (200X).

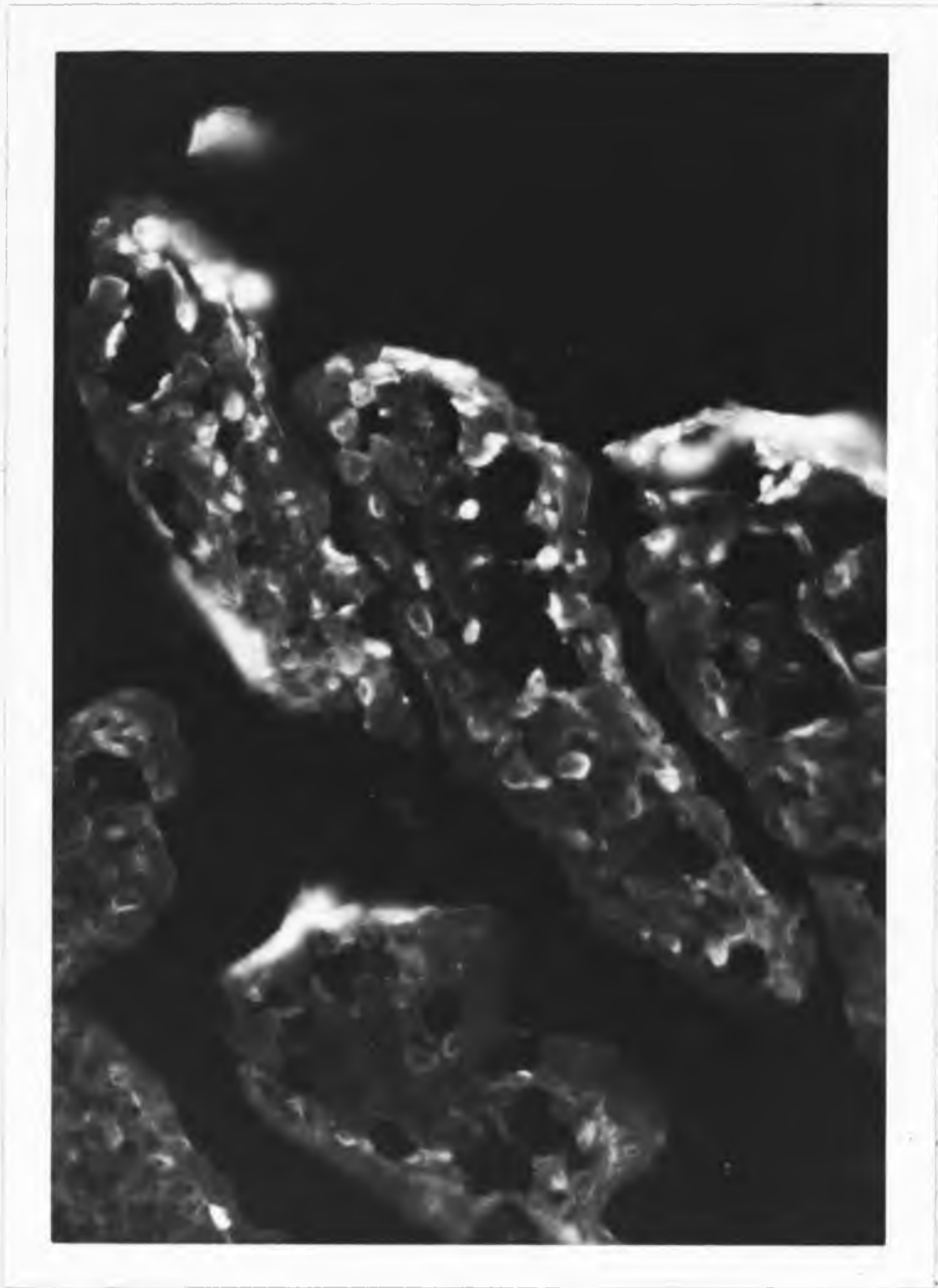

Figure A.9. Lambda Carrageenan Treated Guinea Pig Colon (200X).

REFERENCES

Anderson, W., Carrageenan: Structure and Biological Activity, Canadian Journal of Pharmaceutical Sciences, 2: 81-90, 1967.

Anderson, W. and J. G. C. Duncan, The Anticoagulant Activity of Carrageenan, Journal of Pharmacy and Pharmacology, 17: 647-654, 1965.

Atkinson, R. M., L. Jenkins, E. G. Tomich and E. A. Woolett, The Effects of Some Anti-Inflammatory Substances on Carrageenan Induced Granulomata, Journal of Endocrinology, 25: 87-93, 1962.

Beattie, I. A., W. R. Blakemore, E. T. Dewar and M. H. Warwick, A Study of Orally Administered Degraded Carrageenan in the Baboon, Food and Cosmetic Toxicology, 8: 257-266, 1970.

Black, W. A. P., W. R. Blakemore, J. A. Colquoun and E. T. Dewar, The Evaluation of Some Red Marine Algae as a Source of Carrageenan and of Its Kappa and Lambda Components, Journal of The Science of Food and Agriculture, 16: 573-585, 1965.

Davies, G. E., Inhibition of Guinea Pig Complement In Vitro and In Vivo by Carrageenan, Immunology, 6: 561-568, 1963.

Davies, G. E., Inhibition of Complement by Carrageenan: Mode of Action, Effect on Allergic Reactions and on Complement of Various Species, Immunology, 8: 291-299, 1965.

DiRosa, M., Biological Properties of Carrageenan, Journal of Pharmacy and Pharmacology, 24: 89-102, 1972.

DiRosa, M., J. P. Giroud and D. A. Willoughby, Studies of the Mediators of the Acute Inflammatory Response Induced in Rats in Different Sites by Carrageenan and Turpentine, Journal of Pathology, 104: 15-29, 1971.

Engster, M. and R. Abraham, Cecal Response to Different Molecular Weights and Types of Carrageenan in the Guinea Pig, Toxicology and Applied Pharmacology, 38: 265-282, 1976.

FDA, Federal Register, 15434, August 2, 1972.

Goldstein, J. L., R. G. W. Anderson and M. S. Brown, Coated Pits, Coated Vesicles and Receptor-Mediated Endocytosis, Nature, 279: 679-685, 1979.

Grasso, P., M. Sharratt, F. M. B. Carpanini and S. D. Gangolli, Studies on Carrageenan and Large Bowel Ulceration in Mammals, Food and Cosmetic Toxicology, 11: 555, 1973.

Johnson, G. D., E. J. Holborow and J. Dorling, Handbook of Experimental Immunology, Chapter 15, 1978.

Johnston, K. H. and E. L. McCandless, The Immunological Response of Rabbits to Carrageenans, Sulfated Galactans Extracted from Marine Algae, Journal of Immunology, 101: 556-562, 1972.

Kliegman, R. M., W. B. Pittard and A. A. Fanaroff, Necrotizing Enterocolitis in Neonates Fed Human Milk, Journal of Pediatrics, 95 (3): 450-453, 1979.

Moriarty, R. R., N. N. Finer, S. F. Cox, H. J. Philips, A. Theeman, A. R. Stewart and O. A. Ulano, Necrotizing Enterocolitis and Human Milk, Journal of Pediatrics, 94(2): 295-296, 1979.

Pitman, K. A., L. Goldberg and F. Coulston, Carrageenan: The Effect of Molecular Weight and Polymer Type on Its Uptake, Excretion and Degradation in Animals, Food and Cosmetic Toxicology, 14: 85-93, 1976.

Richer, S. M. and E. L. McCandless, Demonstration of Intracellular Carrageenan by a Fluorescent Antibody Technique in Granuloma Tissue, The Journal of Histochemistry and Cytochemistry, 20: 991-994, 1972.

Santulli, T. V., J. N. Schullinger, W. C. Heird, R. D. Gongaware, J. Wigger, B. Barlow, W. B. Blanc and W. B. Berdon, Acute Necrotizing Enterocolitis in Infancy: A Review of 64 Cases, Pediatrics, 55: 376-387, 1975.

Schwartz, H. V. and R. W. Kellermeyer, Carrageenan and Delayed Hypersensitivity. II. Activation of Hageman Factor by Carrageenan and Its Possible Significance, Proceedings of the Society of Experimental Biology and Medicine, 132: 1021-1024, 1969.

Selner, J. C., D. A. Merrill and H. N. Claman, Salivary Immunoglobulin and Albumin: Development During the Neonatal Period, Journal of Pediatrics, 72: 685, 1968.

Steele, P. R. M. and J. R. Lowes, A Histopathological Study of the Effects of Intravenously Administered Lambda Carrageenan in the Mouse with Particular Reference to the Macrophage System, British Journal of Experimental Pathology, 60: 358-365, 1979.

Virnig, N. L. and J. W. Reynolds, Epidemiological Aspects of Neonatal Necrotizing Enterocolitis, American Journal of Diseases of Children, 128: 1974.

Walker, W. A. and K. J. Isselbacher, Uptake and Transport of Macromolecules by the Intesting (Possible Role in Clinical Disorders), Gastroenterology, 67: 531-550, 1974.

Watt, J. and R. Marcus, Ulcerative Colitis in the Guinea Pig Caused by Seaweed Extract, Journal of Pharmacy and Pharmacology, 21: Suppl. 187-188, 1969.

WHO Technical Representative Service, No. 445, Annex 5, 1970.

Williams, G. A., Histological Study of the Connective Tissue Reaction to Carrageenan, Journal of Pathology and Bacteriology, 73: 557-563, 1957.