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CARRAGEENAN

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Summary

Upon ingestion, carrageenan is rapidly excreted in the feces, absorption occurring only slightly (5, 8, 10). Carrageenan is toxic to macrophages (1, 12, 44), and, as a consequence, causes suppression of delayed hypersensitivity reactions (44). The activation of Hageman factor by carrageenan (43) results in procoagulant activity (43), increased vascular permeability (43), and liberation of kinin (41, 43). Carrageenan inhibits the activity of complement by interfering with the binding capacity of C'1 (11, 15, 16). The sulfate content of carrageenan determines the degree of anticoagulant (3, 25, 26) and antipeptic (4, 35, 46) activity that it exhibits. The viscosity of the gastrointestinal mucus is increased by formation of a carrageenan-mucoprotein complex (6). This increases the protection afforded by the mucous coat by decreasing the penetration of gastric juice into the gastro-duodenal mucosa (6). Eventual precipitation, or sloughing-off, of this complex decreases the protection afforded (33).

There is a difference in the effect of degraded and undegraded carrageenan on test subjects. As a rule, the degraded carrageenan derived from Eucheuma spinosum results in cecal and colonic ulceration of susceptible species. Undegraded carrageenan derived from Chondrus crispus has shown an effect upon the colon of susceptible species as well as a marked depression of the growth rate. As a rule, though, the undegraded form is less toxic than the degraded form.

Degraded carrageenan exerts no toxic effect on gerbils when administered for six months as 2000-3000 mg/kg/day in the diet (28). When administered in drinking water over a period of seven months, degraded carrageenan showed no toxic effects for female rats at a dosage of 9000-10000 mg/kg/day; however, over the same period, growth rate of male rats was depressed at a dosage of 6000-7000 mg/kg/day (27). Guinea pigs are quite susceptible to degraded carrageenan. Guinea pigs administered degraded carrageenan in drinking water for six months at a dosage of 60 mg/kg/day exhibited no toxic effects; however, guinea pigs dosed at 600 mg/kg/day for two weeks exhibited severe ulceration of the cecum and/or colon (28). At a level of 1500 mg/kg/day for twenty to thirty days, severe ulceration of the colon was observed in test guinea pigs (47). Rabbits, likewise, revealed severe ulceration of the gastrointestinal tract when, over a period of three months, degraded carrageenan was provided in drinking water at 70 mg/kg/day (48, 49). Monkeys provided 667 mg/kg/day degraded carrageenan for fourteen weeks exhibited adverse colonic changes. At higher dosages, there was a fall in the hematocrit, number of circulating erythrocytes, and a reduction in hemoglobin level (28).

Undegraded carrageenan also causes adverse effects in susceptible species. Gerbils are unaffected by undegraded carrageenan administered for six months at a level of 2000-4200 mg/kg/day (28). Several studies on rats have shown that they are moderately refractory to undegraded carrageenan in the diet. Undegraded carrageenan provided for six months in drinking water at levels of 600-900 mg/kg/day (28) and 4000 mg/kg/day (32) resulted in no effects to test rats. One study lasting five months revealed that carrageenan (of an unknown nature - presumably undegraded) depressed growth of rats when fed at a level of 10,000 mg/kg/day in the diet (27). Undegraded carrageenan derived from Eucheuma spinosum provided in drinking water to guinea pigs for twenty to thirty days at a dosage of 300 mg/kg/day resulted in severe ulceration of the cecum and colon (47). Guinea pigs given 800-1100 mg/kg/day for one year of an undegraded carrageenan derived from Chondrus crispus suffered no ill effects (28). Monkeys provided undegraded carrageenan (derived from Chondrus crispus) in drinking water for a period of twelve weeks at a dosage of 1300 mg/kg/day experienced a consistent fall in the number of circulating erythrocytes after nine weeks (28).

Following is a tabulation of selected studies:

Animal	Time	Substance	No Toxic Eff. Level mg/kg	Min. Toxic Eff. Level mg/kg	Ref.
Gerbil	6 mo	DC	2000-3000		28
Gerbil	6 mo	UC	2000-4200		28
Rat	7 mo	DC	9000-10000		28
Rat	7 mo	DC		6000-7000	28
Rat	5 mo	UC	5000	10000	27
Rat	6 mo	UC	4000		32
Rat	6 mo	UC	600-900		28
Guinea Pig	1 yr	UC	800-1100		28
Guinea Pig	20-30/dy	UC		300	47
Guinea Pig	20-30/dy	DC		1500	47
Guinea Pig	2 wk	DC		600	28
Guinea Pig	6 mo	DC	60		28
Guinea Pig		DC		1500	28
Rabbit	3 mo	DC		70	48
Rabbit	3 mo	DC		70	49
Monkey	14 wk	DC		667	28
Monkey	12 wk	UC		1300	28

Recent studies indicate that the sodium salt of carrageenan has a fetotoxic effect on rats and mice (21); the calcium salt has a similar effect (20). Although at this time there is no conclusive evidence, both the sodium and calcium salts give indications that they may affect skeletal development in the fetus of several test species (20, 21). Abnormalities in skeletal maturation noted in the fetuses from female hamsters treated with sodium carrageenate indicate the probability of a true teratogenic effect in this species (21).

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Chemical Information

I. Nomenclature

A. Common names
Chondrus
Carrageenan
Irish moss
Pig-wrack
Pearl moss
Killeen
Salt rock moss
Irish gum

B. Chemical names
Sulfate ester of a polysaccharide of galactose

C. Trade names
Viscarin
Burtonite
V-40-E
SEA-KEM

D. Chemical Abstracts Registry numbers

Carrageenan	PM9000071
Carrageenan, sodium	977007707
Carrageenan, calcium	977052144
Carrageenan, potassium	977050193

II. Empirical Formula

Not applicable

III. Structural Formula

Carrageenan is a polymer composed of galactose units. The basic structure can be written (A-1-(1→3)-B-(1→4)-)n where A is:

3,6-anyhdro-alpha-D-galactopyranose 2-sulfate,
alpha-D-galactopyranose 2,6-disulfate, and/or
alpha-D-galactopyranose 6-sulfate.

and B is:

beta-D-galactopyranose 4-sulfate,
beta-D-galactopyranose, or
beta-D-galactopyranose 2-sulfate (39).

Figure I shows a proposed structure of carrageenan (40).

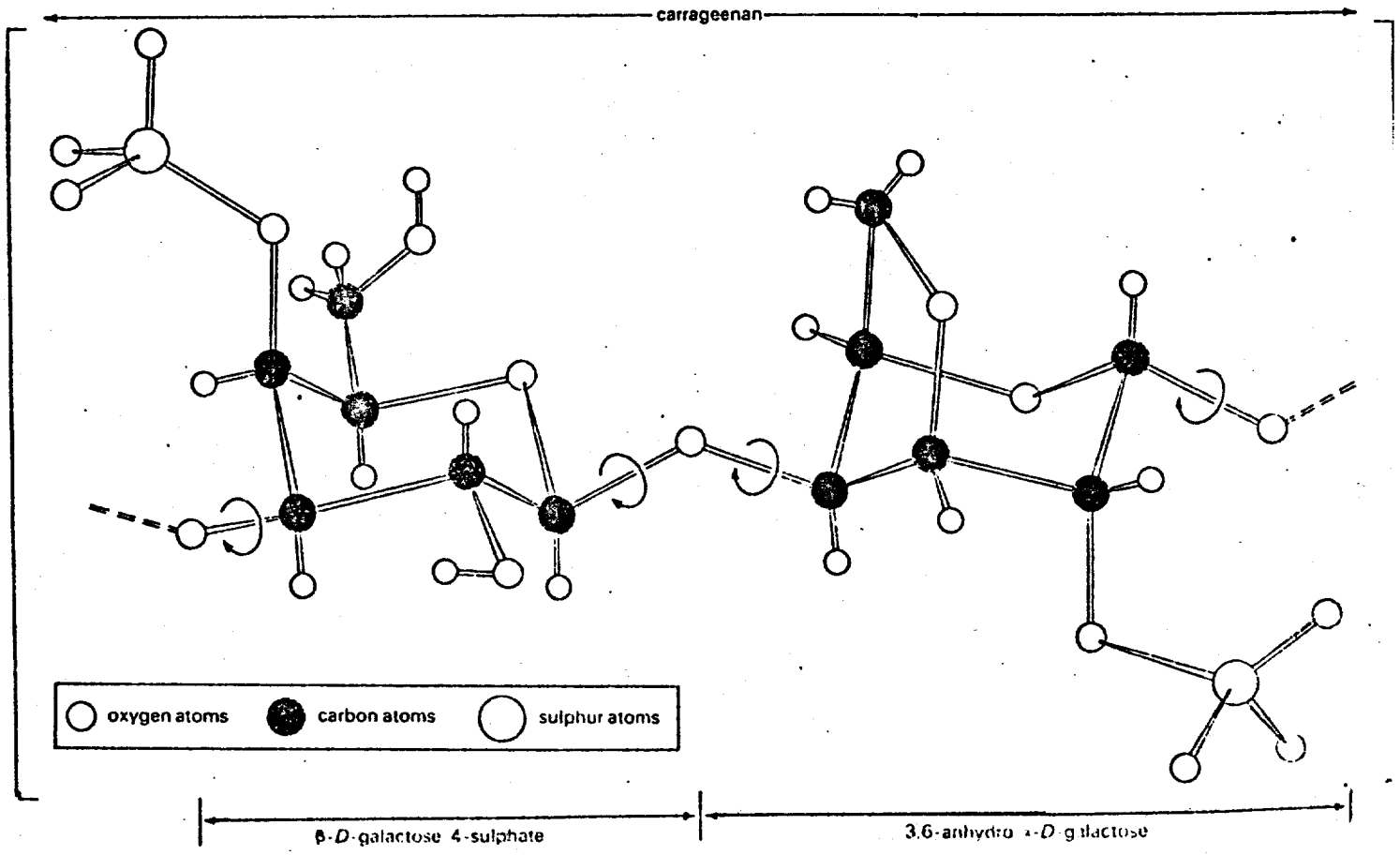
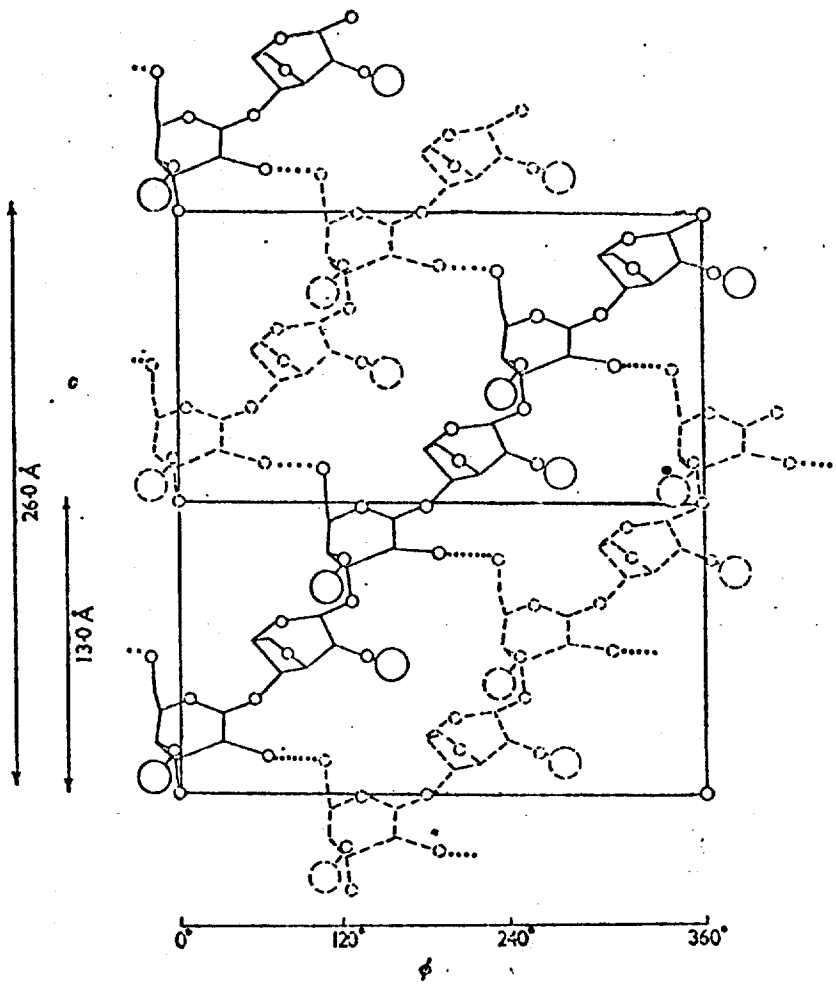


Fig. I. Disaccharide unit of carrageenan.

It has been proposed (2) that carrageenan consists of pairs of polysaccharide chains arranged as double helices. There are three disaccharides in one turn of helix with a repeating distance of 26.0 A (iota) or 26.4 A (kappa). Figure II shows the proposed arrangement of polysaccharide chains.

Fig. 11. The proposed arrangement of polysaccharide chains in iota carrageenan.



IV. Molecular Weight

Molecular weights of the various carrageenans range from 100,000 to 800,000 (40). The commercial material is estimated to be about 120,000 (39).

V. Specifications

Food Chemical Codex	
Ash (total)	Not more than 35%
Ash (Acid-insoluble)	Not more than 1%
Loss on drying	Not more than 12%
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm (0.0003%)
Heavy metals (as Pb)	Not more than 10 ppm (0.001%)

VI. Description

A. General characteristics

1. Carrageenan - a yellowish to colorless, coarse to fine powder, practically odorless with a mucilaginous taste
2. Calcium carrageenan - a tan powder
3. Sodium carrageenan - a light tan powder

B. Physical properties

1. Soluble in water 1g/ 100 ml at 80 degrees and on cooling forms a reversible gel
2. Sparingly soluble in formamide and readily soluble in hydrazine

C. Stability

Dry powdered carrageenan products slowly lose their strength over 6 to 12 months, probably by simple depolymerization.

VII. Analytical Methods

Carrageenan occurs in many commercial products. The official method of analysis involves the treatment of a dairy product with trichloroacetic acid, centrifuging the precipitate and treating the supernatent with alcohol and saturated NaCl solution to precipitate the gum. Solutions can be treated directly with alcohol. The gum precipitate is washed and purified by reprecipitation.

When the gum is isolated, it can be determined qualitatively by a systematic analytical scheme such as one devised by Ewart and Chapman (19). This method is based on precipitation reactions with calcium chloride, sodium hydroxide, barium hydroxide and lead acetate. This scheme distinguishes between pectin, alginate, gelatin, starch, carboxymethylcellulose, methylcellulose, and several other gums, including carrageenan, tragacanth, agar, locust, karaya, ghatti. The same gums can be identified by a similar scheme (7) using the reagents: iodine-potassium iodide in zinc chloride solution, alcoholic iodine, ruthenium red, and sulfuric acid.

The isolated gum can also be identified by its infrared spectrum (7, 37).

A quantitative method for the determination of carrageenan in milk products was developed by Graham (24). The mixture is digested with papain, clarified with celite and the hydrocolloid precipitated from the digest with cetyl pyridinium chloride. The carrageenan content of the isolated complex was determined by the phenol-sulfuric acid method.

VIII. Occurrence

The U.S. Federal Register describes the food additive carrageenan as the refined hydrocolloid prepared by an aqueous extraction from members of the families: Gigartinaceae and Solieraceae of the class Rhodophyceae, including Aeodes orbitose, Chondrus canaliculatus, Chondrus crispus, Chondrus ocellatus, Eucheuma cottonii, Eucheuma edule, Eucheuma muricatum (E. spinosum, E. denticulatum), Gigartina acicularis, Gigartina asperifolia, Gigartina canaliculata, Gigartina decipiens, Gigartina pistillata, Gigartina radula, Gigartina stellata (G. mamillose), Gloiopeltis coliformis, Gloiopeltis furcata, Gloiopeltis tenax, Gymnogongrus norvegicus, Gymnogongrus patens, Iridaea laminarioides, Iridaea capensis (Iridophycus capensis), and Iridaea flaccida (Iridophycus flaccidum). However, Chondrus crispus is the most important source in the United States, and Chondrus and Gigartina in Europe (30, 45).

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Biological Data

I. Acute Toxicity

Animal	No.	Sex	Substance	Dosage mg/kg	Measurement (IG)	Reference
Rabbit	25	M	Na^{+} Carr.	4670-5430	LD_{50}	21
Rabbit	25	F	Na^{+} Carr.	4670-5430	LD_{50}	21
Hamsters	30	M	Na^{+} Carr.	7530-8470	LD_{50}	21
Hamsters	30	F	Na^{+} Carr.	7530-8470	LD_{50}	21
Rats	25	M	Na^{+} Carr.	5650-6250	LD_{50}	21
Rats	25	F	Na^{+} Carr.	5650-6250	LD_{50}	21
Mice	25	M	Na^{+} Carr.	8730-9670	LD_{50}	21
Mice	25	F	Na^{+} Carr.	8730-9670	LD_{50}	21
Rabbit	25	M	Ca^{++} Carr.	2280-3000	LD_{50}	20
Rabbit	25	F	Ca^{++} Carr.	2280-3000	LD_{50}	20
Hamsters	25	M	Ca^{++} Carr.	6180-7320	LD_{50}	20
Hamsters	25	F	Ca^{++} Carr.	6180-7320	LD_{50}	20
Rats	30	M	Ca^{++} Carr.	5140-5660	LD_{50}	20
Rats	30	F	Ca^{++} Carr.	5140-5660	LD_{50}	20
Mice	25	M	Ca^{++} Carr.	8710-9590	LD_{50}	20
Mice	25	F	Ca^{++} Carr.	8710-9590	LD_{50}	20

II. Short-term Studies

Mice

Short-term studies of mice are mentioned briefly in the literature. A six month study on mice revealed no effect from carrageenan (32). Another report states that mice develop ulcerative changes in the colon, though changes are minimal in comparison to other species, when given degraded carrageenan in drinking water (33).

In neither study was sufficient data given to make an assessment of the effects of carrageenan.

Gerbils

Three groups of five male gerbils were fed as follows: one group was fed 5% degraded carrageenan (der. Eucheuma spinosum) in the diet, one group was fed 5% carrageenan (der. Chondrus crispus), and a control group was fed a stock diet. Body weights and food consumption were recorded weekly. Stools were examined for consistency and the presence of occult blood (28).

After six months, neither test group showed any toxic effects. Hematests were negative for all animals. The average carrageenan intake was almost 3550 mg/kg/day for animals fed degraded carrageenan, and the intake for those animals fed carrageenan was about 4200 mg/kg/day. By 23 weeks, the intake of both test groups was close to 2000 mg/kg/day (28).

Rats

Hawkins and Yaphe examined the effects of carrageenan on the growth of rats. Eleven groups of rats (five male, five female) housed individually were fed diets containing 30% polysaccharides (2%, 5%, 10%, 15%, or 20% carrageenan + starch) or a similar regimen of cellulose and starch. One control group was fed 30% starch. Rats were fed 8 g of ration for six days and 15 g/day thereafter until day 149. On days 27, 98, and 148, feces were collected for 24 hours preceding and a carrageenan determination was run on them. On day 28, 99, and 149, feces and urine were collected for nitrogen determination (27).

Carrageenan was 90-100% excreted at levels of 2-20%; however, carrageenan did result in a greater adverse effect upon growth rate than cellulose. The effect became more evident as the concentration of carrageenan increased. There were no reported ill effects at the 2% or 5% level, questionable effects on growth at the 10% level, and marked effects upon growth at the 15% and 20% levels. No other noticeable effects were reported (27).

These experiments are difficult to judge as a result of the unreported nature of the carrageenan fed the test animals. The effect of carrageenan on nitrogen absorption simply was not determinable from the limited data obtained.

A group of twenty (ten male, ten female) rats was given 5% degraded carrageenan (der. E.spinosum) in distilled water that was changed daily. A group of ten (five male, five female) rats was used as control. Body weights were recorded weekly and stools checked for consistency and occult blood. Two male and two female test animals were sacrificed at one and three months for histologic examination. Hematologic studies were executed at two weeks and at seven months (28).

Although females showed normal food consumption and growth, growth of males was only 82% that of the control animals. Fluid intake was increased. Soft stools were observed within 24 hours; occult blood was observed the first week in some rats and by the fourth in most. Hematologic indices were normal at two weeks and at seven months (28).

No significant alteration of the gastrointestinal tract was noted either grossly or microscopically with the exception of a single rat which had several small ulcers in the distal colon (28).

Initial dosage was 9700 mg/kg/day in males and 9000 mg/kg/day in females. By fourteen weeks, males were receiving 6400 mg/kg/day. Thereafter, the levels stabilized at 6000-7000 mg/kg/day for males and 9000-10000 mg/kg/day for females (28).

Maillet reports that rats given carrageenan at a dosage of 4000 mg/kg/day for six months gave no evidence of mucosal erosions (32); but Marcus and Watt report that rats given degraded carrageenan in water develop ulcerative changes in the colon (33).

Neither worker provides sufficient data to compare the conflicting reports and make an assessment on their relative merit.

A group of twenty (ten male, ten female) rats was given 1% carrageenan (der. Chondrus crispus) in sterile distilled water that was changed daily. A group of ten (five male, five female) rats served as control. Fluid consumption was recorded daily; body weight was recorded weekly. Stools were checked for consistency and tested for occult blood. Hematologic studies were run at four months. Four (two male, two female) rats were sacrificed at six months for gastrointestinal examination (28).

Food consumption and growth rate were normal. Stools were softer than those of control but were consistently negative for occult blood. Hematologic indices were normal at four months. Initial intake was 1300 mg/kg/day for males and 1800 mg/kg/day for females. By six months, this had stabilized at 600-900 mg/kg/day for both males and females (28).

Guinea Pigs

Adult male albino guinea pigs housed individually were fed a stock diet supplemented daily with cabbage and were separated into three groups of ten each. One group had 1% carrageenan (der. E.spinosum) in the drinking water, another group had 5% degraded carrageenan (der. E.spinosum) in water, and a third had only water. The water was supplied ad lib and daily intake was recorded. During the test, stools were examined by the hematest method for occult blood. After twenty to thirty days, animals were sacrificed and the gastrointestinal tract removed and prepared for gross and microscopic examination (47).

In the group receiving 1% carrageenan, overall incidence of ulceration was 80%. The ulcerative colitis incidence of animals receiving the 5% degraded carrageenan was 100%. The control group was unaffected (47).

The lesions produced are not readily visible unless the bowel is emptied of feces and transmitted light examination is made. This may account for the fact that these ulcerative changes have not been reported before 1969.

Maillet reported that both degraded and native carrageenan fed to guinea pigs resulted in an erosion of the mucosa, but that this was not ulceration (32).

A group of twenty (ten male, ten female) guinea pigs was given a 5% solution of degraded carrageenan (der. E.spinosum) in sterile distilled water that was changed daily. A second group of ten (five male, five female) animals was used as control. Body weights were determined weekly. Stools were checked for consistency and occult blood. Dead animals were autopsied (28).

These animals consistently lost weight. Stools were fluid by 48 hours, and occult blood was demonstrable in half the test subjects within two weeks. Autopsies of animals that died showed that multiple petechiae were present in the cecum with scattered nodules in the cecal mucosa. There were dense accumulations of macrophages and fibroblasts in the lamnia propria and submucosa. Ulcerations of the epithelium were present (28).

Another group of 33 female guinea pigs, 25 experimental and eight control, was divided into three groups and dosed as follows: eight given .02% degraded carrageenan (der. E.spinosum), eight given 0.2% degraded carrageenan, and nine given 2% degraded carrageenan. The 2% dosage was discontinued at two weeks. Body weights were recorded two or three times during the first, second, and third weeks and weekly thereafter. Fluid consumption was recorded daily. Stools were checked for consistency and tested for occult blood. All guinea pigs that died were autopsied (28).

Animals receiving .02% and 0.2% degraded carrageenan in their drinking water maintained normal growth, food consumption, and appearance. Stools were also normal, and no occult blood was found. However, at the 2% level, animals rapidly lost weight; stools were semi-fluid and positive for occult blood in two days. Four animals died, four were sacrificed, and one lived and recovered after a six month period. Guinea pigs at this 2% level exhibited erosion of the cecum and/or colon within sixteen days in four of six animals; nodules were observed in the cecal walls of five animals. Two of the animals were unsuitable for histologic examination because of advanced autolysis (28).

A group of fifteen (five male, ten female) guinea pigs was given 1% carrageenan (der. C.crispus) in drinking water for one year. Body weights were recorded weekly. Stools were checked for consistency and occult blood. Nine animals were sacrificed for histologic examination at irregular intervals from two to eight months (28).

These animals remained normal in appearance and behavior. Stools were normal; occult blood tests were negative. No gross abnormalities were found at autopsy, but numerous macrophages (Perls' positive) were observed in the lamnia propria of the cecal sections. The colon was histologically normal. The level of dosage was 800-1000 mg/kg/day (28).

Rabbits

Twenty male California rabbits, five to a group, were housed individually and separated into a control and three test groups receiving respectively 0, 0.1%, 1%, and 5% aqueous solutions of degraded carrageenan (der. E.spinosum) for drinking water. These dosage levels were equivalent to 0, 70, 800, and 1400 mg/kg/day and were received ad lib, the records of fluid consumption being kept daily. Test subjects were weighed and occult blood checked weekly. The experiment ran six to twelve weeks. At twelve weeks, the rabbits were sacrificed and the colon examined for ulcerative lesions (48).

All animals at the 1400 mg/kg/day level developed diarrhea by the seventh day, rapidly lost weight, and showed severe ulceration of the colon. All animals at the 800 mg/kg/day level showed moderately severe ulceration of the colon, loss of weight, and occult blood in the feces by the third week; however, only a single animal developed diarrhea. Animals at the 70 mg/kg/day level gained weight at a rate of about one-sixth that of the control group. Occult blood was demonstrable in the feces by the tenth week in three of five animals. These animals had multiple ulcers of the colon (48).

Eighteen young adult male California rabbits housed individually were separated into a control and two test groups of six each. The test groups were provided degraded carrageenan (der. E.spinosum) in drinking water at a daily average intake of 0, 70, and 800 mg/kg/day. Water consumption was determined daily; occult blood and weight were determined weekly. At the end of three months, the rabbits were sacrificed and the colons removed, washed, fixed and thoroughly examined. Microscopic examinations of the colonic sections were performed (49).

Ulcers of the colon were present in all animals at the 800 mg/kg/day level and in half of those at the 70 mg/kg/day level. These ulcers were complicated by the development of hyperplastic mucosal changes bearing a close resemblance to the polypoidal lesions which occur in ulcerative colitis in man. These polypoidal formations were the dominant feature in one-third of the rabbits at the higher dosage and were observable in one-half of the rabbits at the lower dosage (49). These hyperplastic glandular mucosal formations showed no evidence of precancerous change over the three month period (49).

Monkeys

Following is a table giving the nature and duration of the exposure of rhesus monkeys (*Macaca mulatta*) to carrageenan:

Group	Sex	Time (wk) On test	Time (wk) Recovery
Control	M	-	-
	M	-	-
	F	-	-
	F	-	-
2% Degraded Carrageenan Derived from *Eucheuma Spinosum*	M	10	24
	M	10	24
	M	11	0
	F	10	20
	F	10	24
	F	7	0
1% Degraded	M	14	0
	F	14	0
0.5% Degraded	M	14	0
	F	14	0
1% Undegraded Carrageenan Derived from *Chondrus Crispus*	M	11	0
	M	10	11
	M	10	11
	F	7	0
	F	10	11
	F	10	11
Dose-ranging Undegraded	M	12	0
	M	12	0
	F	12	0
	F	12	0

III. Long-term Studies

Mice

The first long-term feeding study involving carrageenan was reported by Nilson and Wagner. Male and female weanling mice were taken in groups of ten and housed individually. The mice were fed carrageenan (SEA-KEM) as 1%, 5%, 15%, or 25% dry weight in the diet; ten animals were reserved as control. The experiment lasted 24 months. No intermediate data were obtained on the mice; however, a necropsy was performed on dead animals where possible. The following table shows the survival rate (38):

		Survivors (Months)							
Diet Designation	Number	3	6	9	12	15	18	21	24
Control	10	10	9	9	7	5	4	1	0
1%	10	10	10	9	7	6	1	1	0
5%	10	9	9	7	7	6	4	1	1
15%	10	8	6	5	5	5•	4	2	1
25%	10	10	10	8	8	7	6	5	4

Carrageenan seemed to be free from any deleterious substances adversely affecting growth rate. There was no evidence of injury to parenchymatous organs, directly or indirectly. Mention was made of malignancies occurring; however, no data were included on this aspect. No mention was made of the strain of mice used in the study. Overall, the experiment was very poorly reported.

Rats

Groups of ten male and female weanling rats were housed individually and fed carrageenan (SEA-KEM) as 1%, 5%, 15%, or 25% dry weight in the diet (10). Ten rats were reserved as control. The experiment lasted 24 months. Food and water intake of the rats were examined once a week and weights were taken. A necropsy was performed on all dead subjects where possible. The following table shows survival rate (38):

		Survivors (Months)							
Diet Designation	Number	3	6	9	12	15	18	21	24
Control	10	10	9	9	8	7	7	6	3
1%	10	10	9	9	9	7	6	4	0
5%	10	10	9	8	6	5	4	3	3
15%	10	10	10	10	10	9	9	5	2
25%	10	10	10	9	8	8	7	3	2

Carrageenan seemed to be free from anything adversely affecting the health of rats or the utilization of nutrients. Many lesions of different kinds were noted, but none were consistent among a single group. Malignancy was reported as fairly common in all groups. Tests performed by the carrageenan supplier (Seaplant Chemical Company) during the tenth week of the experiment showed the digestibility of carrageenan to be 50%, regardless of the level fed (38).

This statement is open to challenge. It would be extremely unlikely that carrageenan even remotely approaches this level of digestibility. The experiment is rife with shortcomings.

Data insufficiency included, among other things, unknown strains and sex distribution of test subjects, origin of carrageenan, and, most importantly, unknown incidence of malignancies. The experimental procedure is entirely inadequate; for example, the food and water intake were only recorded once a week. Absolutely no mention is made of numbers of animals examined after death.

Despite repeated citation of this work in the literature, no mention is made of the observed malignancy occurrence. From the results of the study, no valid conclusions may be drawn.

IV. Special Studies

Carcinogenic

Female sixty day old Wistar rats were injected in the left flank with 5 ml of a 1% (W/V) carrageenan solution in a 0.9% saline base. Three days later, rats were injected in the right flank with 1.5 g caproylethyleneimine or acetylethyleneimine in 0.15 ml of arachis oil, and on the seventh day, the same amount was injected in the right flank. Because of the toxicity oof these compounds, dosages were staggered to minimize loss of animals. Although the object of the experiment was to show the carcinogenic nature of the imines, the significant result was obtained from the group of 43 rats injected with carrageenan and used as the control (13).

In this group, 39 of 43 survived 400 days. Of these, six developed sarcomas on the left side within 650 days and eleven within 825 days. None developed sarcomas on the right side. Expected incidence of sarcoma is given as cases/1000 survivors, and is reported as 7.8 and 21.3/1000 at 650 and 825 days respectively. Actual incidence in this experiment is 154 and 344/1000 at 650 and 825 days respectively. This means that the incidence of sarcoma in the group injected with carageenan is 19.6 times the expected level at 650 days and 16.2 times the expected level at 825 days (13).

Surviving monkeys from the group fed carrageenan were used in the dose-ranging studies in which increasing doses of 1% carrageenan from 50 mg/kg/day to 1250 mg/kg/day were given to the test animals by day 33. Treatment at 1250 mg/kg/day was continued for 51 days and animals were sacrificed. All doses were calculated on an individual basis and were administered in a food slurry (28).

Eventually, all of the test subjects were sacrificed (barbiturate overdose), and a thorough autopsy was performed on each animal. Meticulous examination was made of the gastrointestinal tract. Throughout the testing regimens, observations were made on stool consistency, and hematest for occult blood was performed at least three times a week (28).

Monkeys given 2% degraded carrageenan did not gain weight. There was an immediate change in stool consistency (watery) which remained as long as the carrageenan was administered. By the nineteenth day, all animals passed blood consistently until approximately ten weeks after the withdrawal of the carrageenan. There was a fall in the number of erythrocytes and the hematocrit, as well as a reduction in hemoglobin (28).

At the 2% degraded carrageenan level, autopsied monkeys showed multiple hemorrhages of the colon; animals at the 1% level did to a lesser extent. Prolonged administration of 2%, 1%, and 0.5% degraded carrageenan/day produced adverse effects in the colon of all test subjects (28).

Monkeys given 1% carrageenan in drinking water seemed to be more or less comparable in all respects to the control animals except that there was a consistent fall in the number of circulating erythrocytes after nine weeks (28).

In the dose-ranging study, three of four monkeys showed various liver alterations (28).

Man

A preparation of degraded carrageenan (der. E.spinosum), Ebimar, has been used in France for over fifteen years as a chemotherapeutic agent for peptic ulcer. Bonfils reports that two hundred patients receiving Ebimar at a dosage of 5 g/day for six months to two years were monitored closely, and radiological examination was performed at intervals of not greater than four months. None showed any signs of ulcerative colitis (10).

Teratogenic (calcium carrageenate)

Mice

Groups of adult female CD-1 mice (27, 27, 25, 29, 28, 40) housed individually and provided food and water ad lib were given by oral intubation dosages of 0, 150 (aspirin), 10, 45, 470, and 900 mg/kg/day of calcium carrageenate respectively on day 6 through day 15 of gestation. Body weights were recorded on day 0, 6, 11, 15, and 17 of gestation; animals were observed daily for appearance and behavior. On day 17, all dams were subjected to caesarian section, and number of implantation sites, resorption sites, and live and dead fetuses was recorded. Body weight of live pups was also recorded. Fetuses were examined for gross, congenital abnormalities; one-third underwent detailed visceral examination (10x), and two-thirds underwent examination (alizarin red 5-KOH) for skeletal defects (20).

The administration of calcium carrageenate caused an increase in the number of resorptions and/or fetal deaths in utero that was dosage dependent. There was also a corresponding decrease in the number of live pups and a reduction in pup weight. The increased incidence of missing sternebrae and incomplete skull closure may be indicative of a retardation of skeletal maturation. Calcium carrageenate has been shown to be definitely fetotoxic for mice at dosage levels of 470 and 900 mg/kg/day; however, unequivocal teratogenicity of this substance was not established by this experiment (20).

Hamsters

Groups of adult female golden hamsters (30, 30, 26, 26, 27, 30) housed individually and provided food and water ad lib were given by oral intubation dosages of 0, 250 (aspirin), 40, 100, 240, and 600 mg/kg/day of calcium carrageenate respectively on day 6 through day 10 of gestation. Body weights were recorded on days 0, 8, 10, and 14 of the gestation period; animals were observed daily for appearance and behavior (food consumption). On day 14, all animals were subjected to caesarian section, and number of implantation sites, resorption sites, and live and dead fetuses was recorded. Live pups were weighed, and fetuses were examined for gross, congenital abnormalities. One-third of each litter underwent detailed visceral examination (10x), and two-thirds underwent examination (alizarin red 5-KOH) for skeletal defects (20).

There was no clearly significant effect of the calcium carrageenate on hamsters. There was some evidence of delayed skeletal maturation (missing sternebrae and incomplete ossification of extremities); however, this evidence was equivocal (20).

Rats

Groups of adult female Wistar rats (25, 30, 26, 24, 25, 28) housed individually and provided food and water ad lib were given by oral intubation dosages of 0, 250 (aspirin), 40, 100, 240, and 600 mg/kg/day of calcium carrageenate respectively on day 6 through day 15 of gestation. Body weights were recorded on day 0, 6, 11, 15, and 20 of gestation; animals were observed daily for appearance and behavior (food consumption). On day 20, all animals were subjected to caesarian section, and number of implantation sites, resorption sites, and live and dead fetuses was recorded. Live pups were weighed, and fetuses were examined for gross, congenital abnormalities. One-third of each litter underwent detailed visceral examination (10x), and two-thirds were examined (alizarin red 5-KOH) for skeletal defects (20).

Administration of calcium carrageenate to rats caused an increase in the number of resorption sites at a dosage of 600 mg/kg/day. Also at a dosage of 600 mg/kg/day for ten days, there was a decrease in the birth weight of pups. A concurrent retardation in skeletal development was indicated by a dose-dependent increase in missing sternebrae at dosages of 240 and 600 mg/kg/day. Although calcium carrageenate depressed fetal development and caused an increase in early fetal deaths, there was no evidence of teratogenicity for rats (20).

Rabbits

Groups of 15 adult female Dutch-belted rabbits housed individually and provided food and water ad lib were given an injection of 0.4 ml of human chorionic gonadotropin (400 IU) and three hours later were artificially inseminated. From day 6 of gestation to day 18, animals were dosed by intubation 0, 2.5 (6-amino nicotinamide), 3, 10, 60, and 260 mg/kg/day of calcium carrageenate. Body weights were recorded on day 0, 6, 12, 18, and 29; animals were observed daily for behavior (food consumption) and appearance. On day 29, all animals underwent caesarian section and numbers of corporea butea, implantation sites, resorption sites, and live and dead fetuses were recorded. Body weights of live pups were also recorded. Fetuses underwent an examination for gross, congenital abnormalities. Live fetuses were placed in an incubator for 24 hours for the evaluation of neonatal survival. Surviving pups were examined for visceral abnormalities and skeletal defects (20).

There was no clearly discernable effect of calcium carrageenate on either females or offspring (20).

Teratologic (sodium carrageenate)

Mice

Groups of albino adult female CD-1 mice (27, 27, 24, 27, 31, 43) housed individually and provided food and water ad lib were given by oral intubation dosages of 0, 150 (aspirin), 10, 45, 470, and 900 mg/kg/day of sodium carrageenate respectively on day 6 through day 15 of gestation. Body weights were recorded on day 0, 6, 11, 15,

and 17 of gestation; animals were observed daily for appearance and behavior. On day 17, all dams were subjected to caesarian section, and number of implantation sites, resorption sites, and live and dead fetuses was recorded. Body weight of live pups was also recorded. Fetuses were examined for gross, congenital abnormalities; one-third underwent detailed visceral examination (10x), and two-thirds underwent examination (alizarin red 5-KOH) for skeletal defects (21).

The administration of sodium carrageenate to mice caused an increase in the number of resorptions and/or fetal deaths in utero at dosages or 470 and 900 mg/kg/day. There was a corresponding, dosage-dependent decrease in the number of live pups and in the weight of the pups at delivery. There was also an increased incidence of missing sternebrae and of incomplete skull closure. Sodium carrageenate was decidely fetotoxic, but teratogenicity was not established in this experiment (21).

Hamsters

Groups of adult female golden hamsters (30, 30, 26, 26, 27, 30) housed individually and provided food and water ad lib were given by oral intubation dosages of 0, 250 (aspirin), 40, 100, 240, and 600 mg/kg/day of sodium carrageenate respectively on day six through day ten of gestation. Body weights were recorded on day 0, 8, 10, and 14 of the gestation period; animals were observed daily for appearance and behavior (food consumption). On day fourteen, all animals were subject to caesarian section, and number of implantation sites, resorption sites, and live and dead fetuses were recorded. Live pups were weighed, and fetuses were examined for gross, congenital abnormalities. One-third of each litter underwent detailed visceral examination (10x), and two-thirds underwent examination (alizarin red 5-KOH) for skeletal defects (21).

There was delayed skeletal maturation in offspring resulting from the application of sodium carrageenate to pregnant hamsters. This was indicated by an increase in number of fetuses with missing sternebrae, more than 13 ribs, and incomplete ossification of extremities corresponding to increase in dosage. The increase of these abnormalities at all dosage levels indicates a possible teratogenic effect of sodium carrageenate in hamsters (21).

Rats

Groups of albino, adult female Wistar rats (25, 30, 24, 25, 25, 30) housed individually and provided food and water ad lib were given by oral intubation dosages of 0, 250 (aspirin), 40, 100, 240, and 600 mg/kg/day of sodium carrageenate respectively on day 6 through day 15 of gestation. Body weights were recorded on day 0, 6, 11, 15, and 20 of gestation; animals were observed, daily for appearance and behavior (food consumption). On day 20, all animals were subjected to caesarian section, and number of implantation sites, resorption sites, and live and dead fetuses were recorded. Live pups were weighed, and fetuses were examined for gross, congenital abnormalities. One-third of each litter underwent detailed visceral examination (10x), and two-thirds were examined (alizarin red 5-KOH) for skeletal defects (21).

At 600 mg/kg/day for ten days, sodium carrageenate given to pregnant rats resulted in a decrease of birth weight in the offspring. At dosage levels of 240 mg/kg/day and 640 mg/kg/day for 10 days, there was an increase in the number of resorption sites in the female and a slight decrease in the average live litter size. A dosage-dependent increase in missing sternebrae was found. Sodium carrageenate depressed fetal development in the rat and increased early fetal deaths (21).

Rabbits

Six groups of fifteen adult female Dutch-belted rabbits housed individually and provided food and water ad lib were given an injection of 0.4 ml of human chorionic gonadotropin (400 IU) and three hours later were artificially inseminated. From day 6 of gestation through day 18, animals were dosed by intubation 0, 2.5 mg 6-amino nicotinamide, 40, 100, 240, and 600 mg/kg/day of sodium carrageenate. Body weights were recorded on day 0, 6, 12, 18, and 29; animals were observed daily for behavior (food consumption) and appearance. On day 29, all animals underwent caesarian section and numbers of corporea lutea, implantation sites, resorption sites, and live and dead fetuses were recorded. Body weights of live pups were also recorded. Fetuses underwent an examination for gross, congenital abnormalities. Live fetuses were placed in an incubator for 24 hours for the evaluation of neonatal survival. Surviving pups were examined for visceral abnormalities and skeletal defects (21).

The administration of sodium carrageenate caused an increased number of resorptions at three dosage levels (40, 240, and 600 mg/kg/day). There were no other discernable effects on pregnant female rabbits or offspring (21).

Fetotoxic

A 1% aqueous suspension of carrageenan when injected interperitoneally daily between the 11th and 15th day of gestation in NMRI mice produced no fetotoxic effects (22).

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Biochemical Aspects

I. Breakdown

Carrageenan products lose their stability (strength) over a 6-12 month period, probably due to a simple depolymerization. Unheated solutions can tolerate moderate conditions of acidity. However, heating under acidic conditions promotes rapid hydrolysis (degradation) of the carrageenan.

II. Absorption - Disbribution

Insufficient information was obtained on the absorption and digestion of carrageenan to draw any definite conclusions. It appears, however, that carrageenan is only slightly absorbed by most animals, the rat being a possible exception. The carrageenan that is absorbed is localized in the lysosomes (28).

Studies on rats given 1, 5, 15 and 25% carrageenan (SEA-KEM, Type 5) by dry weight in their daily diet indicated that the mean digestibility of carrageenan during the tenth week of the experiment was roughly 50%, irrespective of the level fed. Nilson pointed out that the amount of carrageenan actually used by the animals and that used by bacteria could not be determined from the studies (38).

Degraded kappa-carrageenan from Eucheuma spinosum (C16) was administered orally to guinea pigs in drinking water (1%) over a period of 18 hours, during which time food was withheld. When 298 mg of C16 was consumed, the urine showed only the qualitative presence of C16; using a 5% solution (1850 mg consumed) the urine contained about 0.3 mg/ml at the end of the experiment (18 hrs). In an anti-gastric ulcer experiment, intraduodenal administration of 400 mg C16 in pylorus-ligated guinea pigs gave detectable, but not measurable, urinary metachromasia, indicating a very slight degree of intestinal absorption. Consumption of degraded lambda-carrageenan from Gigartina pistillata (GP-lambda-D2) gave similar results. The intraduodenal administration of 150 mg of GP-lambda-D2 gave no demonstrable urinary metachromasia. Therefore, if absorption of GP-lambda-D2 did occur, it must have been less than 2.5 mg/kg (about 1 mg total per animal). When 400 mg of C16 was administered intraduodenally in addition to an intravenous injection, the measurable urinary carrageenan did not increase over that resulting from the intravenous injection alone. This supports the conclusion that intraduodenal carrageenan is absorbed only in very small amounts. Undegraded carrageenan (from Gigartina pistillata) was not detected in the urine, although the maximum total dose consumed was 158 mg. (The high viscosity of undegraded carrageenan precludes larger oral or intraduodenal doses) (5).

Three mature male baboons (Papio cynocephalus) were starved overnight, sedated, and restrained in a primate chair. Degraded carrageenan (from Eucheuma spinosum) in aqueous solution, was administered as a single dose by stomach tube. Baboon no. 1 received both the high (3 g/kg) and low (240 mg/kg) dose levels with a 14 day rest period between doses. Baboon no. 2 received only the large dose, and no. 3 only the small dose. Urine was collected for 24 hours after dosing and analyzed immediately. The results are given in Table I. At the 3 g/kg level, the urinary excretion of carrageenan amounts to 0.19% of the total dose in baboon no. 1 and 0.10% in baboon no. 2. If it is assumed that all the carrageenan excreted in the urine over 24 hours was contained in the blood volume (60 ml/kg body wt) of the baboon at one time, the maximum concentration of carrageenan in the blood would be 5-10 mg/100 ml. A male baboon was given a single oral dose of 3 mg degraded carrageenan/kg body wt, and 20 ml of blood was withdrawn from the femoral vein at 3 hour intervals and tested for the presence of carrageenan. The experiment was repeated after a seven day rest period. In neither case was any evidence of carrageenan in the blood observed, although the method of testing used could detect a level of 5 micrograms/ml (8).

Table 1. Study of urinary excretion of carrageenan in the baboon

Baboon no.	Baboon weight (kg)	Total carrageenan dose (g)	Dose level (g/kg)	Total volume of urine (ml)	Qualitative metachromasia reaction	Urinary solids (g)	Carrageenan in dry solids (%)	Concn in urine (mg/ml)
1	16.5	50.6	3.1	570	Positive	21.8	0.43	0.168
2	15.7	50.6	3.2	875	Positive	20.9	0.25	0.060
1	15.8	3.8	0.24	410	Negative			
3	18.3	4.2	0.23	1075	Negative			

In an experiment designed to determine whether degraded carrageenan is absorbed from the colon in human patients with ulcerative colitic, 10 such patients were given 10 g of degraded carrageenan daily for 10 days. No evidence of absorption was found of this dosage (10).

Carrageenan is not digested by man (39).

III. Metabolism and Excretion

Elimination of carrageenan appears to be rapid and by means of the feces.

In a study in which male and female rats were given 2-20% carrageenan (SEA-KEM, Type 6) in their daily feed for up to 148 days, 90-100% of the daily carrageenan was excreted in the feces each day (27).

Two groups of five young male rats (Charles River C.D. strain) were given either 5% native or 5% degraded carrageenan (Eucheuma spinosum) in their diet for 10 days. On the tenth day, the 24 hour fecal excretion was collected and analyzed for 3,6-anhydrogalactose, which indicates the level of carrageenan. The rats receiving native carrageenan excreted approximately 80% of the dose in the feces, while those receiving degraded carrageenen excreted about 87%. According to Dewar, the figures obtained, which were lower than he expected, may be partly attributed to errors in measurement (17).

Studies on guinea pigs and on baboons gave evidence of only very slight excretion of carrageenan in the urine, indicating almost exclusive excretion in the feces (8). (See biochemical section, part II).

Bonfils found no evidence of absorption of carrageenan in ulcerative colitis patients, indicating 100% fecal excretion in man (10). (See biochemical section, part II).

IV. Effects on Enzymes and Other Biochemical Parameters.

Carrageenan has been observed to suppress the delayed hypersensitivity reactions of tuberculin-sensitive guinea pigs (44). This effect may be the result of the toxicity of carrageenan for macrophages, acting through osmotic rupture of lysosomes and the subsequent release of intracellular enzymes (1, 12, 44). It has also been suggested that this suppression may be due, at least in part, to the effect of carrageenan on Hageman factor (43).

Ershoff found that the increment in liver cholesterol and liver total lipid induced by cholesterol feeding (1% for 28 days) was largely counteracted by simultaneous feeding of 10% carrageenan (18).

In vitro studies have shown that carrageenan has a dual dose-dependent effect on the recalcified clotting time of normal human plasma (25, 43). At concentrations greater than 0.001 mg/ml, carrageenan exhibits anticoagulant activity (43). The anticoagulant activity is either the result of inhibition of thrombin action (3, 25, 26) or inhibition of fibrin aggregation or polymerization (42). The degree of anticoagulant activity is directly proportional to the sulfate content of the carrageenan (3, 25, 26).

Lower concentrations of carrageenan produce procoagulant activity (43). The maximum procoagulation observed occured at 0.000033 mg/ml, at which level the recalcified clotting time was approximately one half that of the control (43). The procoagulant activity is the result of activation of the Hageman factor (43), which not only promotes blood clotting, but also induces vascular permeability and liberates kinin. That carrageenan can promote vascular permeability (43) and induce kinin release (41, 43) has also been reported.

Carrageenan also inhibits the hemolytic activity of complement, by interfering with the capacity of C'1 (major component of complement) to become bound to sensitized erythrocytes (11, 15, 16).

In vivo and in vitro studies have shown that carrageenan is a competitive inhibitor of peptic activity (4, 35, 46); the antipeptic activity varying with the ester sulfate content (4, 35, 46). Lambda-carrageenan, which has the highest sulfate content, is the best inhibitor of peptic activity, followed by undegraded carrageenan and then kappa-carrageenan (4, 35, 46).

When orally administered, degraded carrageenan forms a complex with the mucoprotein in the mucus lining of the gastro-intestinal tract, affording protection of the mucosa layer by decreasing the penetration of gastric juices into the mucosa (6, 50).

Adverse effects on the colon (ulcerative colitis, mucosal erosion) have been reported as a result of feeding both degraded and undegraded carrageenan to susceptible animals. Complexing of the carrageenan with mucoprotein leads to an increase in viscosity of the mucous coat and eventual precipitation, which finally results in loss of the protective mucous coat and subsequent focal ulcerations. A fall in the number of erythrocytes and the hematocrit and a reduction in hemoglobin was also reported in the monkeys. A teratogenic effect has been observed with the feeding of carrageenan to pregnant animals. (See biological section for details).

V. Drug Interaction

Since carrageenan is a peptic inhibitor it is possible for it to inhibit the digestion of protein in man. However, this is considered to be highly unlikely since the relative concentrations of protein and carrageenan in the normal human diet are not within the observed effected range (46).

Carrageenan forms highly insoluble complexes with tranquilizers and hypotensive agents such as promazine hydrochloride, chlorpromazine hydrochloride, and reserpine, thereby lowering their availability. This reaction is taken advantage of in the formation of sustained release dosage forms of medicinals (23).

The association of carrageenan (carrageenan A8) with substances having an anticholinergic action such as atropine or propantheline show a synergic protective action against Shay's ulcer and experimental cortisonic ulcer (31).

Table I--Preliminary Calculations on Use of Chondrus Extract (Carrageenan) in Regular Foods as Reported by Food Processors in the NAS Survey†

Food Category		Mean Usage Levels			
No.	Name	(No. of Firms Reporting)	Usual Level, %	(No. of Firms Reporting)	Max. Level, %
01	Baked Goods	(*)	0.061	(*)	0.261
05	Milk Products	(5)	0.045	(5)	0.065
06	Cheese	(*)	0.005	(*)	0.006
07	Frozen Dairy Desserts	(6)	0.036	(6)	0.053
08	Processed Fruits, Juices	(*)	0.050	(*)	0.100
09	Fruit Ices, Water Ices	(*)	--	(*)	0.100
15	Condiments, Relishes	(*)	0.100	(*)	0.350
16	Soft Candy	(*)	0.050	(*)	0.100
18	Jams, Jellies, etc.	(5)	0.900	(5)	1.242
19	Sweet Sauces, Toppings	(3)	0.198	(3)	0.303
20	Gelatins, Puddings	(5)	0.173	(5)	0.399
21	Soups, Soup Mixes	(*)	0.420**	(*)	0.420**
23	Beverages--Type I	(*)	0.048	(*)	0.048
24	Beverages--Type II	(*)	0.100	(*)	0.300
28	Dairy Products Analogs	(3)	0.056	(3)	0.097

†These calculations were made on the basis of usage level data submitted by a total of 19 food processors; any data submitted in the NAS baby food survey, in the FEMA survey, or in the brewing, chewing gum, and hard candy surveys have not been completely processed at this time and are not included.

*The asterisk in parentheses indicates that less than 3 firms (i.e., either 1 or 2 firms) reported usage levels in the particular food category.

**These average levels were reported as being used in dry soup mixes, not in the reconstituted soup as usually consumed; therefore, these levels were not used in the calculations in Tables II and III.

D. F. Dodgen
5-31-72

Table II--Preliminary Calculations of the Daily Intake (Mg.) of Chondrus Extract (Carrageenan) per Individual Food Category*

Food Category No.	Food Category Name	Average Intake[1]	Normal High Intake: Usual Use/ High Food[2]	Normal High Intake: Max. Use/ Avg. Food[3]	Very High Intake: Max. Use/ High Food[4]
01	Baked Goods	83.75	124.38	358.35	532.18
05	Milk Products	25.56	69.93	36.92	101.01
06	Cheese	0.65	1.36	0.78	1.63
07	Frozen Dairy Desserts	12.13	24.70	17.86	36.36
08	Processed Fruits, Juices	64.40	128.45	128.80	256.90
09	Fruit Ices, Water Ices	---	---	11.50	23.90
15	Condiments, Relishes	10.90	22.90	38.15	80.15
16	Soft Candy	5.70	12.35	11.40	24.70
18	Jams, Jellies, etc.	99.00	227.70	136.62	314.23
19	Sweet Sauces, Toppings	24.35	48.31	37.27	73.93
20	Gelatins, Puddings	53.46	108.30	123.29	249.77
21	Soups, Soup Mixes	---	---	---	---
23	Beverages--Type I	68.30	153.07	68.30	153.07
24	Beverages--Type II	26.40	57.80	79.20	173.40
28	Dairy Products Analogs	3.25	9.52	5.63	16.49

*Intakes are for adults and children (males + females), ages 2 to 65+ years. Calculations were based on food consumption data for individuals who eat food in these food categories regularly during any 14-day period. Caution: The intakes for the 15 food categories in this table cannot be added to arrive at a combined total daily intake for the substance; for this information, see Table III. All calculations are based on the assumption that the substance is added to all foods in each category at the levels indicated (either usual or maximum, as in Table I).

[1]Usual Level, % (Table I) x Mean Food Consumption (Eaters Only, Table A).

[2]Usual Level, % (Table I) x High Food Consumption (Eaters Only, Table B).

[3]Maximum Level, % (Table I) x Mean Food Consumption (Eaters Only, Table A).

[4]Maximum Level, % (Table I) x High Food Consumption (Eaters Only, Table B).

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Table III--Preliminary Calculations of the Daily Intake (Mg.) of Chondrus Extract (Carrageenan) per Food Category and the Total Dietary*

No.	Name	Average Intake[1]	Normal High Intake: Usual Use/ High Food[2]	Normal High Intake: Max. Use/ Avg. Food[3]	Very High Intake: Max. Use/ High Food[4]
01	Baked Goods	83.69	124.32	358.09	531.92
05	Milk Products	17.78	54.36	25.68	78.52
06	Cheese	0.47	1.18	0.56	1.42
07	Frozen Dairy Desserts	9.22	22.21	13.57	32.70
08	Processed Fruits, Juices	59.15	125.30	118.30	250.60
09	Fruit Ices, Water Ices	---	---	0.70	2.50
15	Condiments, Relishes	8.80	21.20	30.80	74.20
16	Soft Candy	2.90	8.80	5.80	17.60
18	Jams, Jellies, etc.	51.30	159.30	70.79	219.83
19	Sweet Sauces, Toppings	13.46	35.44	20.60	54.24
20	Gelatins, Puddings	35.29	90.83	81.40	209.48
21	Soups, Soup Mixes	---	---	---	---
23	Beverages--Type I	49.92	133.30	49.92	133.30
24	Beverages--Type II	2.50	5.40	7.50	16.20
28	Dairy Products Analogs	0.50	0.84	0.87	1.46
	Totals for all categories	334.98	782.48	784.58	1623.97

*Intakes are for adults and children (males + females), ages 2 to 65+ years. Calculations were based on food consumption data for individuals who may or may not eat food in these food categories regularly during any 14-day period. The intakes for the 15 food categories may be (and have been) added to arrive at a combined total daily intake for the substance. All calculations are based on the assumption that the substance is added to all foods in each category at the levels indicated (either usual or maximum, as in Table I).

[1]Usual Level, % (Table I) X Mean Food Consumption (Total Sample, Table A).

[2]Usual Level, % (Table I) X High Food Consumption (Total Sample, Table B).

[3]Maximum Level, % (Table I) X Mean Food Consumption (Total Sample, Table A).

[4]Maximum Level, % (Table I) X High Food Consumption (Total Sample, Table B).

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GRAS #23 TABLE A - FOOD CONSUMPTION CALCULATIONS MALES + FEMALES
(MRCA MEAN X USDA MEAN)

NAS Regular	Food Category	Mean MRCA Daily Freq. of Eating Eaters Only	Mean MRCA Daily Freq. of Eating Total Sample	Mean USDA Portion Size (Grams)	Food Consumption (Grams Daily) Eaters Only	Food Consumption (Grams Daily) Total Sample
01	Baked Goods	2.66	2.66	51.6	137.3	137.2
05	Milk Products	.62	.43	91.3	56.8	39.5
06	Cheese	.26	.19	49.3	13.0	9.4
07	Frozen Dairy Des	.30	.23	111.6	33.7	25.6
08	Processed Fruits	.72	.66	179.1	128.8	118.3
09	Ices	.15	.01	74.7	11.5	.7
15	Cond/Relishes	.40	.32	27.5	10.5	8.8
16	Soft Candy	.27	.14	42.3	11.4	5.8
18	Jams/Jellies	.31	.16	35.7	11.0	5.7
19	Sweet Sauces	.20	.11	62.2	12.3	6.8
20	Gelatins, Pudd	.22	.14	141.2	30.9	20.4
21	Soups, Mixes	.20	.13	249.5	50.6	31.7
23	Bev. - Type I	.48	.35	293.6	142.3	104.0
24	Bev. - Type II	.17	.02	153.8	26.4	2.5
28	Dairy Prod Anal	.37	.06	15.7	5.8	.9

GRAS #23 TABLE B - FOOD CONSUMPTION CALCULATIONS MALE + FEMALES
(MRCA 90 PCTILE X USDA MEAN)

NAS	Regular Food Category	90 Pctile MRCA Daily Freq. of Eating Eaters Only	90 Pctile MRCA Daily Freq. of Eating Total Sample	Mean USDA Portion Size (Grams)	Food Consumption (Grams Daily) Eaters Only	Food Consumption (Grams Daily) Total Sample
01	Baked Goods	3.95	3.95	51.6	203.9	203.8
05	Milk Products	1.70	1.32	91.3	155.4	120.8
06	Cheese	.55	.48	49.3	27.1	23.6
07	Frozen Dairy Des	.61	.55	111.6	68.6	61.7
08	Processed Fruits	1.43	1.40	179.1	256.9	250.6
09	Ices	.32	.03	74.7	23.9	2.5
15	Con/Relishes	.83	.77	27.5	22.9	21.2
16	Soft Candy	.58	.42	42.3	24.7	17.6
18	Jams/Jellies	.71	.50	35.7	25.3	17.7
19	Sweet Sauces	.39	.29	62.2	24.4	17.9
20	Gelatins, Pudd	.44	.37	141.2	62.6	52.5
21	Soups, Mixes	.43	.34	249.5	106.7	84.9
23	Bev. - Type I	1.09	.95	293.6	318.9	277.7
24	Bev. - Type II	.38	.04	153.8	57.8	5.4
28	Dairy Prod Anal	1.08	.10	15.7	17.0	1.5

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AN EXAMINATION OF THE CYTOTOXIC EFFECTS OF SILICA ON MACROPHAGES*

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Plates 10 to 13

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The fibrogenic effects of silica in man and experimental animals are not yet satisfactorily explained. There is general agreement that in the first instance silica particles deposited in the lung are engulfed by phagocytic cells, which are rapidly destroyed. The cytotoxic effects of silica can be conveniently reproduced in cultures of peritoneal or alveolar macrophages (1), and the relative toxicity of different forms of silica, and of different dusts, on cell cultures agrees with the pathogenicity and fibrogenic potentialities of the dusts in vivo (2, 3).

Silica provides, in fact, a remarkable example of selective toxicity for one cell type by a substance of simple chemical composition and low chemical reactivity. Why particles of silica rapidly kill macrophages whereas other particles of comparable size and surface area (such as carbon particles or diamond dust) are ingested by the cells without harmful effects is a problem of academic interest as well as practical importance in relation to the pathogenesis of silicosis.

Earlier theories of silica toxicity were reviewed by Nagelschmidt (4) and Harington (5). King in his well known "solubility" theory suggested that silicic acid liberated into the tissues from silica particles brings about the deposition of collagen. Later observations did not support this view, as King himself (6) and Nagelschmidt (4) have pointed out. Curran and Rowsell (7) showed that silica particles implanted into the peritoneum in diffusion chambers do not induce any fibrogenic reaction, although silicic acid is liberated from the chambers. Suggestions have also been made that silica dust might damage macrophages as a result of protein adsorption or denaturation, oxidative changes, or interference with enzyme activity such as that of esterases in the cell.

* Preliminary accounts of parts of this investigation were presented at a Symposium on the Pathogenesis of Pulmonary Fibroses (Milan, Italy, December 1964), and at the Second International Symposium on Inhaled Particles and Vapors (Cambridge, England, September 1965).

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None of the evidence in support of these suggestions has been very convincing, and Vigliani and Pernis (8) were led to formulate an autoimmune causation of silicosis. However, extensive investigations have failed to provide any evidence that silica is directly involved in immune reactions, either cellular or humoral, or that an autoimmunity is responsible for the fibrogenesis (9). It now seems likely that if any immune responses occur in silicosis they may be incidental; for example, secondary to infection of the damaged lungs.

Hence the mechanism by which silica particles kill macrophages is still an open question. Recognizing the similarities between the uptake of silica dust and other particles, e.g. bacteria (10), or inert materials, for example Triton WR 1339 (11), we have been led to an alternative explanation of the phenomenon. According to this view, all particles are taken into phagocytic cells in an essentially similar fashion. The cell membrane is invaginated and the particles are enclosed in phagosomes. Primary lysosomes become attached to the phagosomes and discharge their enzymes into them. Provided that the membranes of the secondary lysosomes so formed remain intact, the cells are unharmed. However, if the enclosed particles can react with the lysosomal membranes and make them permeable, enzymes and particles will rapidly escape into the cytoplasm and damage the cells. Thus attention is focused upon interaction of the particles with lipoprotein membranes of phagosomes and of other biological systems. We have elsewhere (12) presented evidence that silica can interact with proteins and phospholipids and lyse red blood cells, whereas nontoxic particles do not have these effects. An analysis of the underlying physicochemical processes was also given.

In this paper cytochemical and electron microscopial observations of macrophages after uptake of silica are described. Conventional biochemical methods involving homogenization and assay of enzymes in particulate and supernatant fractions cannot be applied satisfactorily in the presence of silica. Even if added to normal cells in the cold, silica particles produce damage during the course of homogenization. However, Cohn and Wiener (13) have presented evidence that when lysosomal enzymes are released into the cytoplasm a proportion escapes from the cells into the surrounding medium. We have therefore assayed lysosomal enzymes in the medium of macrophages exposed to silica and other substances, and assume that these reflect, though not quantitatively, release of enzymes from lysosomes.

Material and Methods

Cultures of Macrophages.—Most experiments were carried out with mouse peritoneal macrophages. These were obtained by inoculating 3 ml of culture medium containing 10 IU heparin per ml into mice of strain VSBS/NIMR weighing 18 to 20 g. After brief massage the cells were recovered and dispensed in 1 ml volumes, containing 2×10^6 cells, into tubes with small cover slips. Cells were incubated in culture medium (medium 199, Glaxo Laboratories Ltd., Greenford, England, and 0 to 20% calf serum inactivated at 56°C for 1 hr) overnight before exposure to silica. In some experiments rabbit lung, alveolar macrophages prepared as described by Cohn and Wiener (14) were used.

Silica.—Four types of silica, kindly provided by Dr. I. Bergman of Sheffield were used: (*a*) "Snowit" silica particles (× 9947) of size range 15% 2.5 μ, 49% 2.5 to 5 μ, 30% 5 to

7.5 μ, and 5% 7.5 to 10 μ; (*b*) "Fransil" silica (× 5663), uncompressed, consisting of silica particles of diameter up to 0.3 μ; (*c*) Na-OH-etched Fransil (× 5843 from × 5663) of size range 0.1 to 0.5 μ; and (*d*) silica particles coated with aluminium according to Dale and King (15). All samples were heat-sterilized before being added as fine suspensions to culture media. Living cells were examined by time-lapse, phase-contrast cine-micrography in a warm chamber, and also by dark-ground microscopy.

Control Materials.—(*a*) Diamond dust, particle size 2 to 4 μ (Diamond Products, Sales, Ltd., London). This is not fibrogenic. (*b*) Highly purified carrageenan (Seaplant Corporation, New Bedford, Massachusetts). This sulfated polygalactase is fibrogenic (see reference 16, Williams). Polyvinylpyridine-*N*-oxide (P204, U33) was obtained through the courtesy of Professor H. W. Schlipköter, Düsseldorf.

Histochemical Methods.—Acid phosphatase staining by a modified Gomori method (17) was carried out on unfixed cells and cells fixed in 2% glutaraldehyde buffered to pH 7.0 with 0.1 M cacodylate for 10 to 20 min at 4°C. Controls were treated with sodium fluoride (10^{-2} M) to inhibit the enzyme. Indoxylacetate esterase staining was carried out as described by Holt (18). Fluorescence microscopy of cells (17) was performed after incubation of cells with 1 in 10^5 (w/v) acridine orange in culture medium for (½) hr at 37°C, incubation in fresh medium without acridine for 15 min, mounting in fresh medium, and activation with blue-violet light (460 to 500 mμ). Metachromatic staining for intracellular carrageenan was carried out by washing the cells with saline, fixing in 70% ethanol for 5 min, staining in 0.1% toluidine blue in 30% ethanol for 10 min, and mounting in buffered glycerol.

For histochemical recognition of polyvinylpyridine-*N*-oxide, the method of Jancsó (see reference 47, Pearse) has been used.

Electron Microscopy.—Macrophages were cultured in Petri dishes overnight and then treated with Fransil (100 μg/ml) to avoid difficulty in cutting sections, which was experienced when quartz was used. After time intervals from 2 to 24 hr treated cells and untreated controls were gently scraped off the glass with a silicone rubber "policeman", centrifuged, and fixed in Palade's solution (19) at 4°C. For histochemistry, cells were fixed in 2% cacodylate-buffered glutaraldehyde for 10 min at 4°C, washed, incubated with the Gomori reagent for 15 min at 37°C, washed, and postfixed in Palade's solution. Embedding was in Araldite, and sections were stained with lead according to Karnovsky (20).

Enzyme Determinations in Medium.—Cells were incubated in Petri dishes in standard medium which was replaced by medium lacking serum 2 hr before assay. This can maintain healthy cells for at least 12 hr. Acid phosphatase and β-glucuronidase were measured by conventional techniques (21, 22) using *p*-nitrophenylphosphate and phenolphthalein glucuronide as substrates, incubating 0.2 ml samples of medium in 0.1 M sodium acetate buffer, pH 5.0, at 37°C for 1 hr.

OBSERVATIONS

Light Microscopy.—Macrophages were exposed to silica of various types (10 to 100 μg/ml) in culture media and observed by time-lapse, phase-contrast cine-micrography and dark-ground microscopy. Control cells moved about, preceded by ruffled membranes, and passed freely alongside and sometimes over one another. Phase-contrast dense bodies, identified as lysosomes, moved about in a random fashion in the cytoplasm. Only the larger granules were seen in motion pictures, the movement of the smaller granules being so rapid that it was not recorded. When silica particles were added, they were rapidly engulfed. The particles remained in phagosomes in the cells, often in a paranuclear posi-

tion but sometimes in the more distal part of the cytoplasm. Cells that were engorged with large amounts of silica remained fully extended and showed apparently normal mobility. Within 2 hr nearly all the lysosomes were attached to phagosomes containing silica, and by 10 hr very few granules were left intact, although cell movement was unimpaired. When moving pictures were made after 15 hr, some cells were already dead, and the events associated with cell death were recorded. Some cells that were moving suddenly stopped and remained immobile for a while before the processes were withdrawn and the cells rounded up. In other cells there were violent threshing movements of the cytoplasm while the cells became rounded and eventually immobile. During the later stages there was a tendency for the cells to stick to another, and occasionally they fused to form giant cells.

Histochemical Changes.—Control, unfixed macrophages showed virtually no phosphatase staining (Fig. 1). This corresponds with previous observations (17, 23) that if the membranes of lysosomes are intact the substrate does not penetrate and give a reaction. In fixed macrophages, numerous lysosomes of varying size showed phosphatase and indoxylacetate esterase in the cytoplasm (Fig. 2). After uptake of silica, the particles were readily seen in the cytoplasm by phase-contrast microscopy or dark-ground illumination (Fig. 3). In unfixed cells within a few hours numerous lysosomes were seen clustered around phagosomes containing the dust (Fig. 4). Later the reaction product accumulated in dense masses around the silica particles, and by 15 to 18 hr enzyme had diffused into the cytoplasm of many cells, which were often rounded up (Fig. 5). This change progressed until by 30 hr virtually all cells were rounded up, diffusely stained, and dead. Similar changes, somewhat less marked, were seen after 100 μg/ml Fransil.

Vital staining with acridine orange confirmed this picture. In normal macrophages intense orange fluorescence of lysosomes of varying size was seen throughout the cytoplasm. These were sensitive to light and readily coalesced into large masses while under observation. 5 hr after uptake of silica, many of the lysosomes were clustered around the phagosomes containing silica, where they still showed orange fluorescence. Thereafter there was progressively less discrete orange fluorescence and more diffuse green fluorescence around the silica particles and later in the cytoplasm of the cells.

Carrageenan (20 to 200 μg/ml) was rapidly taken up, even in the absence of serum or basic protein, and readily demonstrated in the phagosomes by its metachromatic reaction (Fig. 7). After 4 hr the phagosomes in unfixed cells showed discrete granules of phosphatase reaction product, and by 6 hr there was marked and more or less homogeneous staining of the phagosomes. This picture remained unchanged for about 18 hr, most of the cells still being fully extended. By 30 hr many cells had rounded up and showed diffuse phosphatase staining. The cells were often stuck together, sometimes bridged by metachromatic-staining material.

Cells took up aluminium-coated silica or diamond dust in essentially the same fashion as silica, and phosphatase was discharged into phagosomes in the usual way. However, the great majority of the cells remained fully extended and mobile for at least 30 hr (Figs. 10 and 11). The phosphatase reaction product closely enveloped the particles in the phagosomes (Fig. 6) and did not apparently escape into the remainder of the cytoplasm or nucleus.

Electron Microscopy.—Normal macrophages showed the cytoplasmic structures that have been described by others (14, 24). These include short, filamentous mitochondria with well defined cristae and numerous vesicles and cisternae lined by smooth membranes extending outwards from the Golgi region to the periphery of the cell (Fig. 9). Many lysosomes were present, and could be identified by the accumulation of acid phosphatase reaction product, though it was never possible to stain all of them in any cell. The lysosomes varied in size and form, usually being surrounded by single membranes and having fairly dense, granular contents. Occasional multivesicular bodies were also seen. Ribosomes were for the most part free, although some were membrane associated.

After addition of silica particles (Fig. 8) these were seen in invaginations of the cell membranes and in phagosomes lined by single, or occasionally double membranes (Figs. 12 and 13). Lysosomes were seen closely associated with the phagosomes (Fig. 13), and possibly discharging their contents into the phagosomes. At this stage the cytoplasmic detail was well preserved. By 14 hr in a number of cells silica particles were seen apparently free in the cytoplasm and mitochondria were swollen and rounded (Fig. 14). By 25 hr many of the cells were severely damaged, with silica particles widely scattered through the cytoplasm and organelles no longer clearly identifiable (Fig. 15). In one preparation silica particles were seen in the nucleus. It is worth emphasizing that there were considerable differences in the time taken to induce cytotoxic effects. Some cells showed obvious damage by 14 hr, but even at 24 hr some cells remained in good condition.

Appearance of Lysosomal Enzymes in the Culture Medium.—Control cultures released only very small amounts of lysosomal enzymes into the culture medium even after 3 days, whereas the enzyme content of the cells increases in culture. Within 14 hr of uptake of silica particles, release of acid phosphatase and β-glucuronidase into the medium was demonstrable. Much more was released at 24 hr and less at 30 hr. The latter may reflect destruction or inactivation of enzyme. With diamond dust only small amounts of enzyme were released in the medium at 30 hr. With carrageenan there was some enzyme release at 24 hr but more at 30 hr.

Protective Effects of Polyvinyl-Pyridine-N-Oxide (PVPNO).—Schlipköter and his colleagues (25) found that PVPNO diminishes the fibrogenic effects of quartz in animals and protects cultures of L cells against cytotoxic effects of silica. This protection was confirmed in macrophages, using biochemical

methods of assessing cell integrity (26). We have confirmed that if mouse macrophages are treated with PVPNO considerable protection against cytotoxicity at 24 hr is found. Even if cells are pretreated with 1% PVPNO for 6 hr, followed by exposure to silica in fresh medium lacking PVPNO, considerable protection is seen. The fate of PVPNO and silica in the cells was therefore of interest. Cells were incubated for 6 hr at 37°C with 4% (w/v) PVPNO, washed with fresh medium, and examined with the histochemical technique for PVPNO. Numerous round cytoplasmic bodies containing PVPNO were seen, which suggests that there is uptake of this polymer into macrophage phagosomes in a manner analogous to the previously described uptake of Triton WR 1339 in liver lysosomes (11). If silica was added afterwards in the absence of PVPNO and the cells incubated for a further 2 hr at 37°C, then silica particles were seen in cytoplasmic vacuoles together with PVPNO. Acid

TABLE I

Release of Lysosomal Enzymes from Macrophages into Culture Medium

Measurements of enzyme activity as described in the text at different times after addition of silica or carrageenan.

Time after addition, *hr*	14		24		30	
Enzyme units	Phosphatase	Glucuronidase	Phosphatase	Glucuronidase	Phosphatase	Glucuronidase
Control	0.11	0.04	0.13	0.12	0.16	0.10
Silica, *100 μg/ml*	2.75	2.98	6.58	5.32	1.80	1.13
Silica + PVPNO	0.52	0.33	0.83	0.77	1.34	0.82
Carrageenan, *100 μg/ml*	0.12	0.09	1.03	0.97	3.6	2.76

phosphatase staining of unfixed cells treated with PVPNO alone, or PVPNO and silica, showed staining of the secondary lysosomes, but very little diffuse staining of cytoplasm even at 24 hr. Leakage of lysosomal enzymes into the cytoplasm was also much less marked after exposure to silica and PVPNO than with silica alone (Table I).

DISCUSSION

It is clear that the initial uptake by macrophages of toxic particles or substances (such as silica or carrageenan) is not noticeably different from that of nontoxic particles (diamond dust or aluminium-coated silica). In both cases engulfment and formation of phagosomes, with discharge of lysosomal enzymes into phagosomes, follows essentially the same pattern. However, the subsequent events are markedly different. Cells that have taken up diamond dust remain healthy for at least 30 hr, whereas cells that have taken up silica quite rapidly succumb. Histochemical studies of the cells with engulfed nontoxic particles

show that lysosomal enzymes remain confined within the phagosomes, and only very small amounts of these enzymes are released into the medium. After uptake of silica, diffuse cytoplasmic staining of lysosomal enzymes is seen and the enzymes are detectable in the surrounding medium. Electron micrographs show that silica particles are at first confined within phagosomes whereas later they are distributed throughout the cytoplasm. About the time that silica particles are seen in the cytoplasm, other morphological changes are apparent. Early changes include swelling and rounding of mitochondria and eventually there is complete disintegration of cytoplasmic structure.

These observations suggest that the integrity of the lysosomal membrane plays a large part in determining whether an ingested particle is toxic. Any particle that can react with constituents of the membrane so as to make it permeable may bring about release of lysosomal enzymes into the cytoplasm. There is evidence that other procedures which make lysosomal membranes permeable, such as exposure to bacterial toxins (27) or treatment with visible light after dyes are taken up by lysosomes (28), can have damaging effects on cells. These may be aggravated by direct interaction of the released particles with cytoplasmic constituents.

This interpretation of silica toxicity is supported by observations that silica can react with protein, phospholipid, and artificial or natural biological membrane systems whereas nontoxic particles such as diamond dust do not react in this way. We have recently presented evidence that the main reaction likely to occur under physiological conditions is hydrogen donation from the phenolic hydroxyl groups of silicic acid in the formation of complexes with hydrogen acceptors such as suitable oxygen or nitrogen atoms in living tissues. Such reactions with secondary amide (peptide) groups of proteins are demonstrable experimentally (12), and these could account for interaction of silicic acid with protein films (29) and the denaturation of proteins and rendering them susceptible to tryptic digestion as described by Scheel and his colleagues (30). However, stronger interactions are demonstrable between hydrogen donors and phosphate ester groups of phospholipids (12). The fact that silicic acid and silica particles are hemolytic (12, 31), whereas nontoxic particles are not, shows that silica can induce permeability changes in biological membrane systems, presumably as a result of interaction with phospholipid or protein.

The protective effects of PVPNO are readily explained on the same basis, because PVPNO is a powerful hydrogen acceptor and readily forms hydrogen bonds with silicic acid (12). Silicic acid or silica particles in the presence of PVPNO are much less hemolytic than in the absence of the polymer (12, 31). Hence it is not surprising that if silica particles and polymer are ingested together by macrophages, the cells are protected. When erythrocytes are treated with PVPNO and washed, there is only slight protection against silica hemolysis (12, 31), so the polymer does not remain associated with red cell membranes.

However, macrophages, unlike red cells, ingest PVPNO, so that the substance can remain in phagosomes which fuse with those containing silica. This is not surprising, since Gordon et al. (32) found that lysosomes prelabeled with colloidal iron fused with others enclosing DNA-protein coacervates labeled with colloidal gold. Likewise, we have observed that if macrophages are exposed first to asbestos and then after some hours to silica, the asbestos and silica particles are demonstrable within the same phagosomes. Thus PVPNO can coat the silica particles before they have time to damage phagosomal membranes. It is worth noting that the silica particles when first ingested are covered with a layer of denatured plasma protein (as can be shown by agglutination with appropriate antisera, see reference 8). Presumably this layer has to be digested away before the silica particles can react with the phagosomal membrane. Silica particles coated with lipid or protein show less interaction with membranes than free particles (31). If silica is added to cells in the presence of high concentrations of serum, its cytotoxic effect is significantly retarded.

The effects of carrageenan will be discussed elsewhere, but it appears that they are similar to those of silica although the cytotoxic effects on macrophages are more slowly exerted than those of silica. The highly acidic groups of the polymer may not be involved in their toxicity (33), but the mechanism of action remains to be fully analyzed.

We have found that silica particles released by death of macrophages have virtually undiminished cytotoxicity when added to fresh cultures of macrophages. Hence in vivo a cyclical killing of these cells would be expected. The relationship between death of macrophages and the fibrogenic effects of silica also remain open to analysis. Suggestive results were obtained by Curran and Clark (34), who implanted agar or egg white into the peritoneum of rats. These brought about death and dissolution of phagocytic cells in the vicinity, and within 4 days there was a well defined fibroblastic reaction around the dying phagocytes with laying down of connective tissue fibres. We have found that repeated subcutaneous injection of frozen and thawed macrophages from the same inbred mouse strain induces local deposition of fibrous tissue. It would thus appear that death of macrophages provides sufficient stimulus for mobilization of fibroblasts and fibrogenesis, although which components of macrophages are involved, and whether there is local transformation of connective tissue cells into fibroblasts or invasion from outside, remain to be determined.

Observations of other investigators, though not primarily concerned with lysosomes, are consistent with our interpretation of silica toxicity. Rilke and Kessel (35) found no detectable change in fixed preparations of acid phosphatase distribution in cells shortly after uptake of silica, whereas Webster et al. (36) found a decrease in macrophages 48 hr after exposure to silica. The methods used would be unsatisfactory for demonstration of redistribution of enzymes such as we have described, and the late changes could be due to loss of enzymes

through intracellular destruction or passage into the medium. Policard et al. (37) following the cellular reaction after injection of silica into rats, showed uptake into phagocytic cells, including neutrophils. They drew attention to disappearance of neutrophil granules in the process. Since neutrophil granules are lysosomes (38), these findings provide additional evidence of lysosomal changes in cells exposed to silica.

There is evidence that acid phosphatase activity increases in vivo in response to silica administration, both acutely in mesenchymal cells of liver and spleen (39) and in developing silicotic nodules (40). The former could be due to lysosomal enzyme activation, the latter to immigration of cells with high acid phosphatase activity and activation.

If lysosomal enzymes play an important part in silica cytotoxicity and the consequent fibrosis, agents which stabilize lysosomal membranes would be expected to diminish these effects. Cortisone and chloroquine are known to stabilize lysosomal membranes and prevent release of lysosomal enzymes under a variety of conditions (41). There is good evidence that cortisone administration diminishes the fibrogenic effects of silica (42, 43) although it is without effect on established lesions (44). Inhibition of the proliferative response to silica by chloroquine has also been described (45).

Thus the available evidence suggests that lysosomes play an important part in silica cytotoxicity and fibrogenesis, and that it is unnecessary to invoke either an immune response by the host or direct stimulation of fibrogenesis by silicic acid to explain the pathogenesis of silicosis. Clearly other factors, such as infection, will often supervene and complicate the pathological picture. The pathological effects of other substances or particles may be explained on a similar basis, although the detailed manifestations and long term effects may be different. We have found, for instance, that the initial fate of asbestos in macrophages is similar to that of silica, and this is one of the substances apart from silica and carrageenan that is fibrogenic. In another situation, the uptake of microcrystals of sodium urate by phagocytic cells, and the inflammatory response so induced, appears to play an important part in the acute exacerbations of gout (46). These inflammatory effects are likewise reduced by prior administration of cortisone or other drugs. Similar amounts of sodium urate in solution do not damage phagocytic cells or induce inflammation and it seems in the case of both silica and urate that having the offending material in particulate form ensures its uptake by phagocytic cells and concentration in a vulnerable region, the lysosome. A great deal of attention has been given to effects of endotoxin and other substances on lysosomes (see reference 41) but relatively little to the importance of these organelles in the pathogenesis of disease by toxic particles or substances. Further work on this aspect of the problem seems to be warranted.

In the case of silica and urate the major uptake is by phagocytic cells (macro-

phages and polymorphonuclears, respectively), the effects on other cells being secondary and indirect. However, we have indications that other substances such as carrageenan are also taken up by fibroblasts, so that the possibility of direct stimulation of cell multiplication and fibrogenesis, perhaps by a lysosomal mechanism, must be considered. This interpretation is different from the previously held view that silicic acid or other substances act merely as templates on which fibrous tissue is deposited.

SUMMARY

Effects of silica, diamond dust, and carrageenan on mouse macrophages were studied by phase-contrast cine-micrography, electron microscopy, histochemical techniques for lysosomal enzymes and measurements of the release of lysosomal enzymes into the culture medium.

All added materials were rapidly taken up into phagosomes, to which lysosomes became attached. In all cases lysosomal enzymes were discharged into the phagosomes to form secondary lysosomes. Within 24 hr most of the silica particles and enzyme had escaped from the secondary lysosomes and lysosomal enzymes were found in the culture media. Most macrophages were killed by this time. With nontoxic particles (diamond dust, aluminium-coated silica, or silica in the presence of the protective agent polyvinyl-pyridine-N-oxide, PVPNO) ingested particles and lysosomal enzymes were retained within the secondary lysosomes for a much longer time, and cytotoxic effects were considerably delayed or absent altogether.

It is concluded that silica particles are toxic because they are efficiently taken up by macrophages and can then react relatively rapidly with the membranes surrounding the secondary lysosomes. The particles and lytic enzymes can then escape into the cytoplasm, producing general damage, and thence into the culture medium. It is suggested that hydrogen bonding of silicic acid with lipid and protein constituents of the membrane accounts for the induced permeability. Protective agents such as PVPNO are retained in lysosomes and preferentially form hydrogen bonds with silicic acid.

Carrageenan is demonstrable within macrophages by its metachromatic reaction. It brings about release of enzymes from secondary lysosomes, but much more slowly than does silica. Silica released from killed macrophages is as cytotoxic as the original preparation. It is suggested that repeated cycles of macrophage killing in vivo leads to the mobilization of fibroblasts and fibrogenesis characterizing the disease silicosis.

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X-ray Diffraction Studies of Polysaccharide Sulphates: Double Helix Models for κ- and ι-Carrageenans

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APPENDIX

Model Building Calculations for Alternating Polysaccharides

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Rec'd 6/6/70

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X-ray Diffraction Studies of Polysaccharide Sulphates: Double Helix Models for κ- and ι-Carrageenans

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Two polysaccharides have been studied which approximate in structure to alternating copolymers, $(—A—B)_n$, in which B is a residue of β-D-galactose-4-sulphate and A is a residue of 3,6-anhydro-α-D-galactose (κ-carrageenan) or its 2-sulphate (ι-carrageenan). The glycoside linkages are A1→3B and B1→4A. A small proportion of A residues also occur as α-D-galactose-6-sulphate and 2,6-disulphate in the natural polymers but were removed by fractionation or conversion to the anhydride with alkaline borohydride. X-ray diffraction photographs of oriented fibres of salts with various monovalent cations then showed fibre axis repeat distances of 24·6 Å (κ-carrageenan) and 13·0 Å (ι-carrageenan). Very similar models can be proposed for both polysaccharides, based on striking relationships between features in the various diffraction photographs, mathematical derivation of all models with appropriate dimensions and symmetry, and calculation of cylindrically averaged Fourier transforms. They are double helices with three disaccharide residues in a complete turn of each single chain, in 24·6 Å (κ-carrageenan) or 26·0 Å (ι-carrageenan). In ι-carrageenan the second chain is displaced exactly half a pitch from the first.

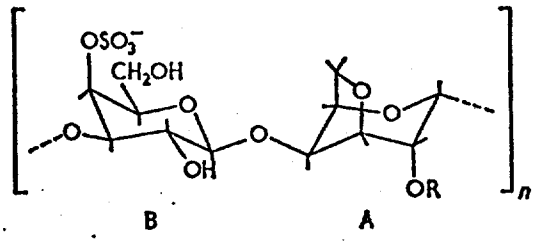
1. Introduction

Certain polysaccharides which we may call "gel-forming polysaccharides" exist in biological situations as swollen networks of hydrated chains, for example in animal and human cartilage, bacterial capsules, and the cell walls of young plants. Their function is partly structural but also involves their elasticity and water holding properties and ability to react selectively with cations. The "tie points" in such networks may be covalent in part, but secondary valence forces between chain segments are also likely to be important, and indeed offer mechanisms for subtle alteration of biological texture. It is therefore likely that a knowledge of polysaccharide tertiary structure, or inter-chain contacts, would be crucial to the understanding of the properties of these systems. Seaweeds provide many polysaccharides which have very striking properties as gels *in vitro* and which have primary structures regular enough to suggest the use of diffraction methods. We have selected two of this group as models in which to study the role of tertiary structure in polysaccharide networks.

Careful selection of source, sometimes followed by subfractionation and/or chemical modification of the native polysaccharide, is necessary to obtain regular structures likely to give interpretable fibre diagrams. Some years ago, before the polysaccharide structures were known, Bayley (1955) made an X-ray study. In so far as comparison

† All enquiries should be addressed to M.M.H. or D.A.R.

is possible, our results are in agreement but the interpretations are different. Our materials were prepared for X-ray diffraction from κ-carrageenan and ι-carrageenan as described in the Methods section, and henceforth referred to as "kappa" and "iota", respectively, although we did not use the crude native polysaccharides. As shown in formula I, these derivatives are regularly alternating polymers of the type $(—A—B)_n$ where B is the β-D-galactose-4-sulphate residue and A is the residue of 3,6-anhydro-α-D-galactose (in kappa) (Anderson, Dolan & Rees, 1968), or its 2-sulphate (in iota) (Anderson, Dolan & Rees, unpublished results).



I

Kappa: R=H
Iota: R=SO3⁻

2. Methods

(a) *Polysaccharide fibres*

ι-Carrageenan was prepared in the laboratory of Marine Colloids, Inc., from carefully handsorted *Eucheuma spinosum*. It was treated (Rees, 1961) with sodium hydroxide–sodium borohydride reagent at 80°C to convert the galactose-2,6-disulphate which represents about one tenth of the 4-linked residues (Anderson, Dolan & Rees, unpublished results), into 3,6-anhydride. Without this step the diffraction photographs of the potassium salt were almost featureless. Kappa was from *Chrondrus crispus* and was a highly purified subfraction described as sample 8 in an earlier paper (Anderson, Dolan, Penman *et al.*, 1968). The polysaccharides were obtained in each salt form by passage of a dilute solution through an ion exchange column in the appropriate state (Amberlite IR 120, analytical grade), followed by evaporation at 35°C under diminished pressure and final freeze drying. A drop of warm aqueous solution, at a concentration to give a soft gel when barely cool (1 to 4%), was placed across the glass beads in a cell (Fuller, Hutchison, Spencer & Wilkins, 1967) and drawn out whilst it set. Constant humidity (about 58%) was maintained by means of a saturated sodium bromide solution and the fibre was removed after 48 hr at 0 to 3°C. Fibre densities were measured by flotation in mixtures of carbon tetrachloride and benzene or carbon tetrachloride and methyl iodide.

(b) *X-ray diffraction*

Photographs were taken with CuKα radiation from a Philips fine focus X-ray tube, collimated by a fine lead glass capillary. The specimen was mounted on a Supper precession camera, with the fibre axis tilted, usually by 10°, out of the plane normal to the X-ray beam. The 'movement' of the precession camera was not used; the film was usually positioned 6·00 cm from the fibre. The intensity distributions were recorded with a Joyce–Loebl microdensitometer.

(c) *Transforms*

Intensity distributions were calculated on a KDF9 computer for various molecular models, according to the formula given by Klug, Crick & Wyckoff (1958) for the cylindrically averaged Fourier transform of a helical structure. Bessel functions up to the ninth order were included and the square of the Fourier transform was calculated at intervals of 0·02 in ξ.

3. Diffraction Photographs

(a) *Comparison of salt forms*

Various salts of kappa and iota gave fibres which were examined by X-ray diffraction:

Cation	Degree of orientation Kappa salt	Iota salt
NH_4^+	+	+++
Li^+	none	+++
Na^+	none	+++
K^+	++	+++
Rb^+	++	++
Cs^+	+	+

The degree of orientation in the different salts, as judged by the X-ray photographs, varies with the ability to form a gel, and is generally better in the iota series than in the kappa series. The fibres could be described as semicrystalline (Holmes & Blow, 1965). The best fibres were those of the potassium salt of iota (Plate I).

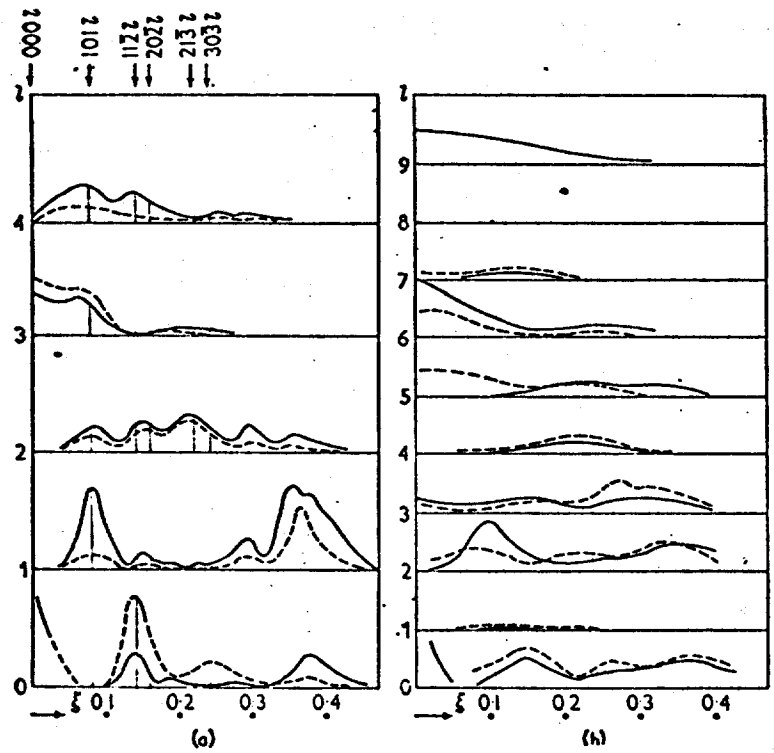


FIG. 1. Examples of intensity distribution in the layer lines (l) of kappa and iota salts. ξ is dimensionless and equal to $2 \sin \theta$ when $l = 0$. On the left are the ammonium and potassium salts of iota (broken lines and full lines), and on the right are the ammonium and rubidium salts of kappa (broken lines and full lines).

The positions of the first few reciprocal lattice points in iota corresponding to a hexagonal unit cell of side $a = 22{\cdot}6$ Å are indicated.

(b) *Fibre axis repeat distance for iota*

All the iota salts have a fibre axis repeat distance, c, of $13{\cdot}0 \pm 0{\cdot}3$ Å, deduced from layer line spacings. The diffraction pattern is consistent with a helical arrangement (Cochran, Crick & Vand, 1952), containing three disaccharide residues per turn of helix; on the meridian there is a diffraction maximum in the third layer but not in any other layer up to the fifth.

(c) *Salts are isostructural*

The fibre axis repeat distance is the same, within a few per cent, for the different salts of iota; the general character of the intensity distributions is the same but there are some marked differences, as shown in Figure 1. At present we attribute these to the different scattering powers of the cations and assume that the polysaccharide skeleton is the same throughout the iota salts or the kappa salts. Difficulties in deducing relative scale factors for the intensity distributions of the various salts have so far prevented us from using the formal isomorphous replacement method for structure solution.

(d) *Fibre axis repeat in kappa and relation of kappa to iota*

The diffraction patterns of the kappa salts are much more diffuse than those of the iota salts; the fibre axis repeat distance is $24{\cdot}6 \pm 0{\cdot}8$ Å, i.e. about twice that of iota. The intensity distributions indicate a close relation to iota; the layers $l = 0, 2, 4, 6$ of kappa are similar to the layers $l = 0, 1, 2, 3$ of iota while the layer $l = 1$ in all kappa salts is very weak or absent. Diffraction on the meridian—present only when $l = 3$, 6 or 9—shows that there are three disaccharide units per turn of this helix.

(e) *Unit cell*

The layer line spacings define the fibre axis repeat distance or the c axis of a unit cell. Within the layer lines the intensity distribution is nearly continuous, but in iota the first few maxima are consistent with a hexagonal cell of side $a = 22{\cdot}6$ Å—as shown in Figure 1. In the zero layer, $10\bar{1}0$ is absent whereas $11\bar{2}0$ is very strong; $11\bar{2}0$ corresponds to the first order of diffraction from the related hexagonal cell of side

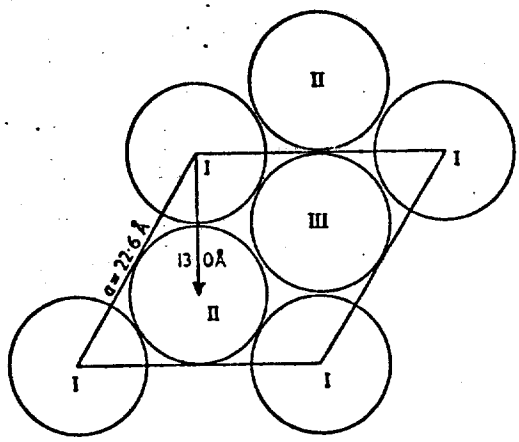


FIG. 2. Cross-section of a hexagonal array of helices of diameter about 13 Å. It is proposed that the helices I, II and III have different displacements in the c-axis direction so that, while the true unit cell has side $a = 22{\cdot}6$ Å, the unit cell seen in this projection has side $13{\cdot}0$ Å.

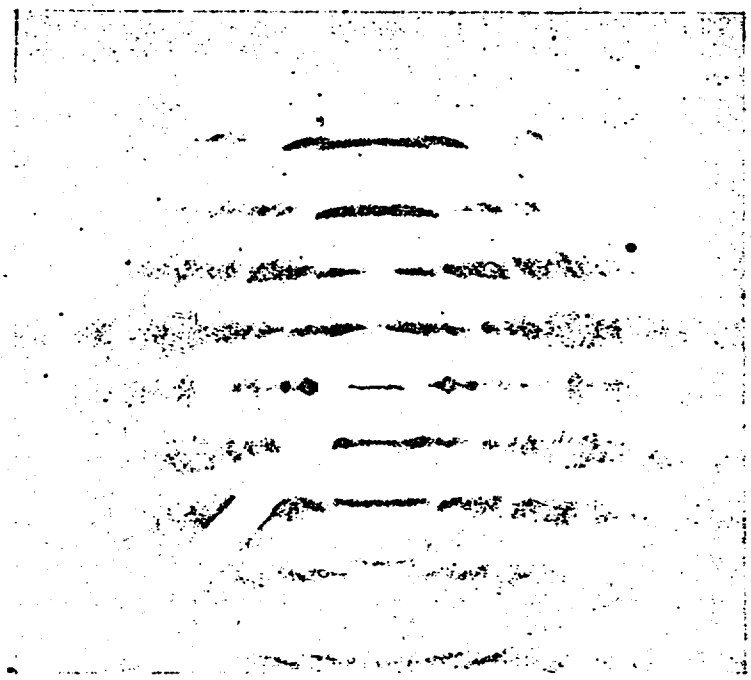


PLATE I. X-ray diffraction photograph of the potassium salt of iota carrageenan. Tilt of fibre was 10°.

[facing p. 88

TABLE 1

Numbers of disaccharide residues per fibre axis repeat from the measured densities

	Iota salts		Kappa salt
	NH_4^+	K^+	K^+
Molecular weight of disaccharide + 15% water	575	623	488
Observed density (g cm^{-3})	1·57	1·72	1·62
Hence volume per disaccharide (Å^3)	608	601	500
Unit cell *a* (Å)	22·6	22·6	*ca.*20
c (Å)	13·0	13·0	24·6
Unit cell volume/(3 × vol./disaccharide) = number of disaccharides per fibre axis repeat	3·15	3·19	5·68

$a/\sqrt{3}$=13·0 Å (Fig. 2) and suggests a hexagonal arrray of helices, each of diameter approximately 13 Å. Helices I, II and III in Figure 2 must have different displacements in the *c* direction. A similar cell with *a* approximately 20 Å is consistent with the kappa photographs.

The number of disaccharide units per fibre axis repeat in the above cells can be deduced from the measured densities, as shown in Table 1. Uncertainties in the water content and cell dimensions are sufficient to account for the discrepancy between the figures deduced and 3·0 or 6·0.

4. Double Helix Models

We propose that in both kappa and iota there are pairs of polysaccharide chains arranged as double helices. In each chemically distinct polysaccharide chain there are three disaccharides in one turn of helix, with a repeating distance of 26·0 Å (iota) or 24·6 Å (kappa). In iota, the disaccharide units of the second (parallel) helix are located exactly half way between those of the first helix giving a fibre axis repeat distance of 13·0 Å as shown in Figure 3. In kappa we do not yet know the exact relationship of the two helices except that it is not such as to halve the fibre axis repeat distance.

We first proposed this double helix model after consideration of the general features of the diffraction pattern and the close relation of kappa and iota, and then found that a model with acceptable stereochemistry in the polysaccharide chain could be built to conform to these requirements. For iota, a single helix with three disaccharide residues per turn, repeating after 13·0 Å, was still a formal possibility but should give a much larger intensity on the first layer line than that observed. A single helix structure for kappa, to maintain its similarity to iota, would need to have six disaccharide residues in *two* turns of a helix repeating after 24·6 Å; chemically identical groups would then be in stereochemically different situations. The double helix structure accounts very well for the weak first layer line in kappa.

Subsequently, we systematically explored polysaccharide chain conformations for iota which could give either single or double helices of the right dimensions and which had acceptable stereochemistry and van der Waals contacts (see below). The acceptable conformations could be represented by five single and six double helices, and

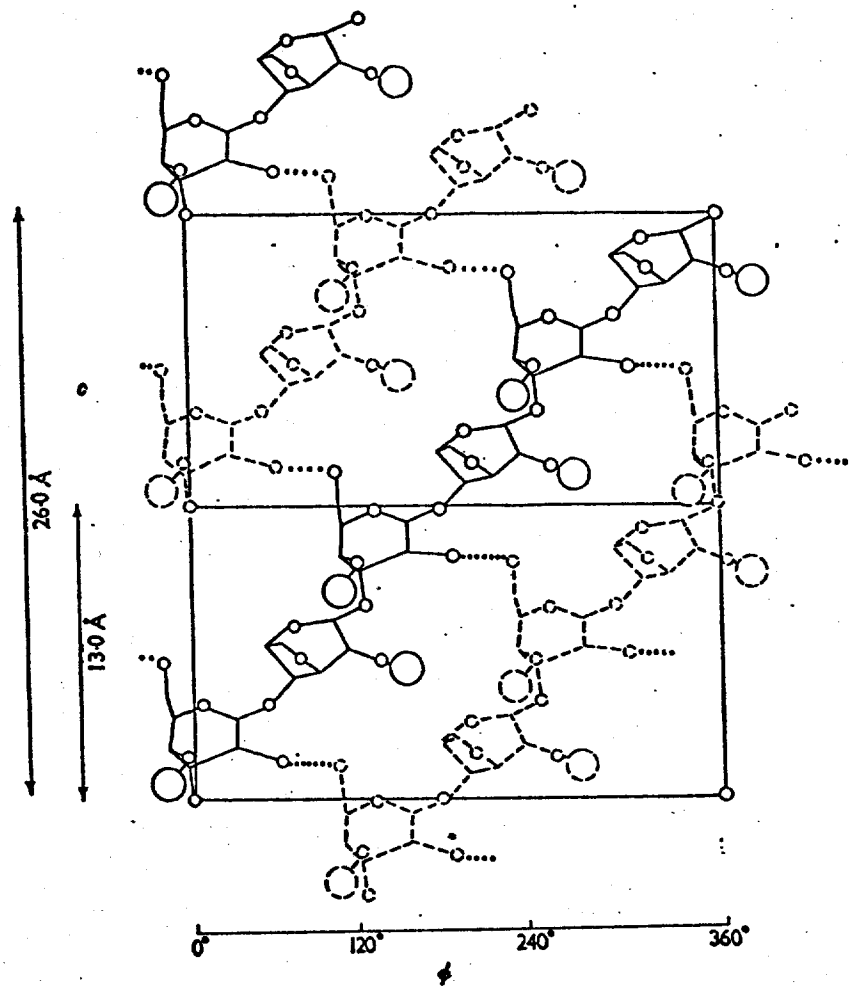


Fig. 3. The proposed arrangement of polysaccharide chains in iota. Two chemically distinct chains, represented by full and broken lines, respectively, and each having three disaccharide residues in one turn of helix of pitch 26·0 Å, are twisted together to form a double helix with a translation period of 13·0 Å. The structure is represented as a projection on the surface of a cylinder which has then been opened out. Small circles represent oxygen atoms and large circles are SO_3^- groups.

The co-ordinates correspond to model H (see text); the proposed O(2) .... O(6) hydrogen bond is shown by dotted lines.

sulphate ester group positions were calculated for each. Intensity distributions were calculated for all of these and compared with the Li^+ salt of iota. The agreement between observed and calculated intensity was poor for all the single helices, better for most of the double helices, and quite promising for models F, G and H (Table 2). Two of the intensity distributions are illustrated in Figure 4. The best model appeared to be H, which was essentially the same as the model, 1, derived by a different mathematical procedure and selected as the best according to stereochemical criteria. Inspection of a molecular model built from the calculated co-ordinates suggested a hydrogen bond, perpendicular to the helix axis, between O(6) of one galactose unit and O(2) of its partner in the second strand. The only hydroxy-groups in the repeating

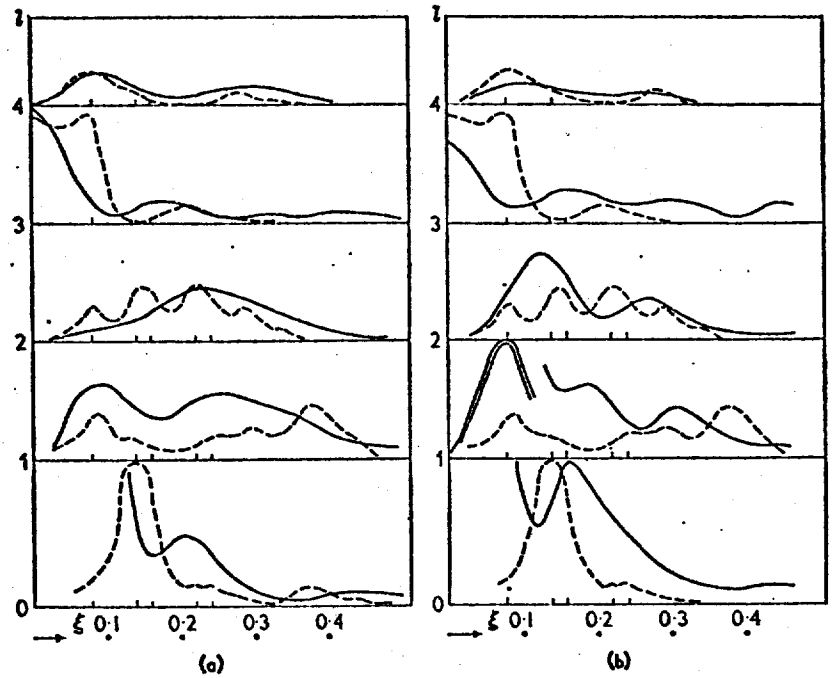


FIG. 4. Comparison of observed intensity distribution (broken lines) in the lithium salt of iota with the intensity distribution calculated for (a) the double helix model H, (b) the single helix model A. The latter model is rejected, mainly on account of the first layer disagreement. (The positions of reciprocal lattice points are indicated as in Fig. 2.)

unit (see formula I) would then be engaged in hydrogen bonding, making this helix seem very plausible on structural grounds. There is some experimental evidence for such a hydrogen bond (D. A. Rees & F. B. Williamson, unpublished results). Oriented films of kappa and iota show a continuous and almost featureless absorption in the O—H stretching region of the infrared spectrum but, after deuteration, there is a sharp band at 3330 cm^{-1} which corresponds to a hydrogen bond of length 2·8 to 2·9 Å. The very pronounced perpendicular dichroism of the band and its resistance to deuteration are consistent with an inter-strand hydrogen bond buried in the helix interior. Its presence in the spectra of kappa and iota is a further indication of similarity between the two conformations.

We therefore conclude that the structure is a double helix with a conformation close to H; this is illustrated in Figure 3, and the model 1, which is almost identical, is illustrated in Figure 5. The sulphate groups are on the outside of the double helix and cannot be as rigidly fixed by stereochemical requirements as can most other atoms of the polysaccharide chain. However, the average contribution of the sulphate groups to the calculated intensity distribution is about half the total. This distribution is therefore very sensitive to the sulphate positions and not as sensitive as we would wish to the conformation of the polysaccharide chain. We expect that the hexagonal array of double helices is held together by interactions between the sulphate groups and cations. We are now trying to establish these positions and hope that this will lead to better agreement between the observed and calculated intensity distributions.

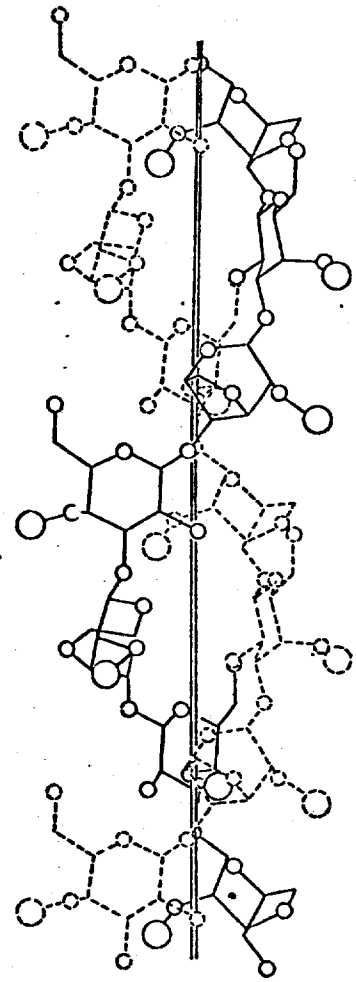


Fig. 5. Model I for the iota double helix. Full lines and dotted lines represent chemically distinct chains. Small open circles represent oxygen atoms and larger circles SO_3^- groups. See Fig. 3 for the position of O(6) .... O(2) hydrogen bonds.

5. Model Building

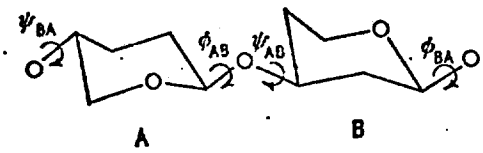
The feasibility of a double helix was confirmed for iota with ball-and-spoke and with Corey–Pauling–Koltun space filling models. Two independent computer methods were then used in an objective and exhaustive survey, each based on a different mathematical treatment and on a separate set of reasonable stereochemical assumptions and residue co-ordinates.

It is assumed that each monosaccharide residue, and the bond angle at each glycosidic oxygen, has a fixed geometry which can be predicted from analogous crystal structures. To define a regular chain conformation it is then only necessary to fix the four angles of rotation, ϕ_{AB}, ψ_{AB}, ϕ_{BA} and ψ_{BA}, which are shown in formula II†. This approach has been much used in conformational analysis of macromolecules

† For consistency with the literature, we must unfortunately use ϕ in three different ways: (i) ϕ_{AB}, ϕ_{BA} are torsion angles about glycosidic bonds; (ii) ϕ_i are angles between vectors and bonds (see Appendix); (iii) ϕ is a cylindrical polar co-ordinate.

(Ramachandran, Ramakrishnan & Sasisekharan, 1963; De Santis, Giglio, Liquori & Ripamonti, 1963; Ramachandran & Sasisekharan, 1968; Scheraga, 1968; Brant & Flory, 1965).

The first computer method, "Modelbuild I", was used only to derive a sterically feasible iota double helix; the mathematics will be described elsewhere. All possible



II

combinations of values of ϕ_{AB} and ψ_{AB} were explored and those which led to impossible contacts across the glycosidic linkage, judged by "outer limit" criteria (Ramachandran *et al.*, 1963), were rejected. For each allowed combination of these angles, the values from the diffraction photograph for n (screw symmetry) and h (projected length of the disaccharide residue on the helix axis) were used to fix the remaining parameters, ϕ_{BA} and ψ_{BA}. The conformation was accepted if contacts were satisfactory (i) across the B—A linkage (ii) with a second chain added to form a double helix and displaced from the first by half the translation period. Some of the principles involved have been used before in constructing trial models for cellulose (Jones, 1958), xylan (Settineri & Marchessault, 1965), and amylose triacetate (Sarko & Marchessault, 1967), although each of these structures has only one monosaccharide residue in the chemical repeating unit and therefore the conformation has only two variable torsion angles.

Most of the carrageenan structures were derived by the second procedure, Modelbuild II. The stereochemical assumptions, and conformational maps which show allowed combinations of each ϕ and ψ, have been described (Rees, 1969*a*). These combinations were taken together in all possible ways to compute the helical parameters, n and h, and thus, by interpolation, n and h values to match experiment. The coordinates of the polysaccharide chain were then transferred to helix axes for further calculations. Details are given in the Appendix. All interatomic distances were checked to the fourth residue up and down the chain. Contacts between the two chains in the double helix as proposed for iota were checked; in kappa the screwing of one chain relative to the other must be considered and this was explored with the typical results shown in Figure 6. Sulphate esters have some rotational freedom but Corey–Pauling–Koltun models suggest that this is confined to an oscillation through $\pm 10°$ about a mean position in which the H — C — O — S system is coplanar and the C — H and O — S bonds are eclipsed. The sulphates were placed in this conformation with one of the remaining O — S bonds also in the plane and *anti* to the C — O bond. The bond angles were tetrahedral with S — O bond lengths of 1·60 (to the ester oxygen) and 1·45 Å (to the terminal oxygens).

In the absence of any constraints whatsoever and if the angles were taken in 10° steps, the total number of regular conformations for a carrageenan chain would be about 1,680,000. When the outer limit criteria were imposed with the requirement that $n = \pm 3$ and $h = 8{\cdot}67$ Å, Modelbuild I reduced this number to 25 right-handed and 15 left-handed helices. All the left-handed helices failed to accommodate a second

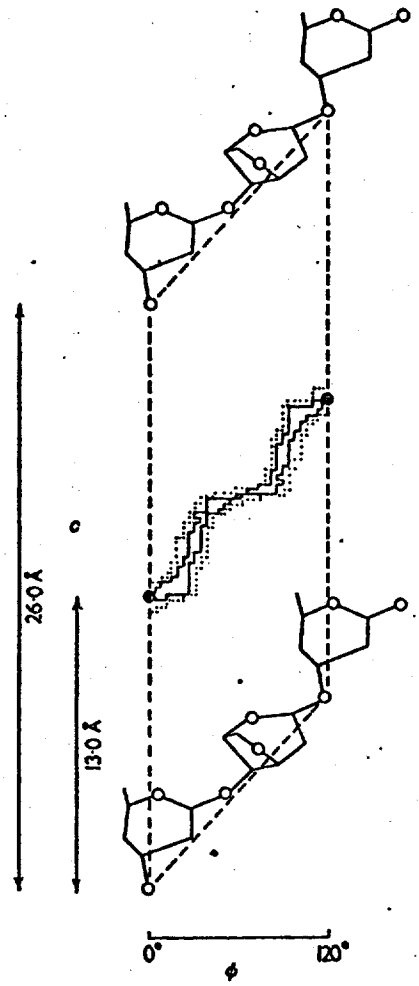


Fig. 6. Computer calculations to show that it is sterically possible for carrageenans to form double helices with the chains in different relative positions. An iota chain is drawn in radial projection (cf. Fig. 3) to correspond to model H with some atoms omitted for clarity. A disaccharide residue, representing a parallel second strand, is moved over the grid inside the large parallelogram defined by the dashed lines, with checking of all relevant contacts to the first chain. Sulphate groups were omitted at this stage; they are on the outside of the double helix and Corey–Pauling–Koltun models show that they have little steric influence on packing within the double helix. Possible positions for the origin atom (O(3) of B) occur in a strip across the middle of the parallelogram and are boxed by solid lines ("fully allowed") and dotted lines ("marginally allowed"). Contact criteria were as used by Rees (1969*a*).

The double helix proposed for iota has the origin of the second chain in the fully allowed position marked thus ○. Allowed areas of about the same size, but differing in shape and position, were also found in the middle of the map when the disaccharide was antiparallel to the first chain.

chain to form a double helix but 17 right-handed helices remained. When calculations were repeated with the allowable contact distances increased by 0·1 Å, all the conformations failed except one, designated "model I".

Using Modelbuild II, systematic variation of ϕ_{AB}, ψ_{AB}, ϕ_{BA} and ψ_{BA} in 20° steps followed by interpolation and checking of remote contacts and contacts between strands, led to 17 iota double helices, all of which had right-handed chains. Comparison

of conformational maps calculated at 10° and 20° intervals showed that no allowed areas were missed by using the larger steps. Conformations which differed by less than 15° in all angles of rotation were grouped and one representative was taken from near the middle of the zone to give six final possibilities (Table 2); model H resembled

TABLE 2

Representative iota conformations derived by systematic exploration of all possibilities using Modelbuild II

Model	Type	Helical parameters		Conformation angles (in degrees)			
				A — B linkage		B — A linkage	
		n	h	ϕ_{AB}	ψ_{AB}	ϕ_{BA}	ψ_{BA}
A	Single	3·00	4·33	110·0	221·0	97·6	142·0
B	Single	3·00	4·33	120·0	218·0	74·5	161·0
C	Single	3·00	4·33	120·0	225·9	103·0	120·7
D	Single	3·00	4·33	101·0	229·5	120·0	123·3
E	Single	−3·00	4·33	123·5	241·0	305·1	180·0
F	Double	3·00	8·67	130·0	191·12	142·75	160·0
G	Double	3·00	8·67	122·3	194·0	160·0	151·3
H	Double	3·00	8·67	139·7	198·2	148·0	140·0
J	Double	3·00	8·67	159·9	204·2	140·0	122·0
K	Double	3·00	8·67	160·0	190·0	119·6	153·4
L	Double	3·00	8·67	171·0	195·8	120·0	137·7

A negative value of n implies a left-handed helix; all other helices are right handed.

model I. The average radii of all these double helices were similar and would allow packing in a unit cell of the type proposed, with space for cations and water; most atoms were within 5 Å of the helix axis with only a few as far out as 7 Å, but no further.

A similar number of kappa conformations could be derived, taking $h = 8{\cdot}20$ Å. Kappa and iota possibilities usually differed by bond rotations of less than 15°, and were therefore similar in their chain contours. Left-handed kappa helices, like the corresponding iota helices, were excluded by their failure to accommodate a second chain even when outer limit criteria were used in the test.

Single helices were also considered for iota, taking $n = \pm 3$ and $h = 4{\cdot}33$; 15 conformations were found initially but seven of these were eliminated because of remote contacts closer than outer limit distances and others were similar enough to be grouped, giving the five final possibilities listed in Table 2, only one of which was left-handed. They had larger radii than the double helices, with many atoms placed 5·5 to 9 Å from the axis, and would be more difficult to pack in a unit cell with the dimensions proposed.

In the maps for inter-chain contacts in various double helices derived for kappa and iota, the chains seemed to be least crowded when symmetry related as proposed for iota (see Fig. 6). In kappa, the chains may be parallel and displaced from the iota arrangement by "screwing", or they might be antiparallel. Enough maps have been calculated to show that adequate space for the polysaccharide chain is available in either case over a range of screw displacements, but further progress is unlikely until the sulphate and cation positions have been deduced.

6. Possible Relation to Physical and Biological Properties

These double helices are the first examples for polysaccharides, although a triple helix has been proposed for an algal xylan (Atkins, Parker & Preston, 1969).

Both κ- and ι-carrageenans occur naturally in the gel state and, after extraction, may form stiff thermally reversible gels in low concentrations in aqueous solution (Percival & McDowell, 1967; Rees, 1969*b*). It has been shown that this gelation involves the cross-linking or close association of two or more chains (Anderson, Dolan, Penman *et al.*, 1968). The similarity between the cation requirements for gelation and fibre orientation by κ-carrageenan suggests the same type of association in each situation. Gelation is not prevented by formal removal of a proportion of either type of sulphate shown in formula I (Painter, 1960; Stancioff & Stanley, 1969) but formal transfer of sulphate from C(4) (as in I) to C(2) of B, or formal insertion of a proportion of galactose-6-sulphate in place of A residues, is inhibitory (Anderson, Dolan, Lawson, Penman & Rees, 1968; Lawson & Rees, 1968; Anderson, Dolan, Penman *et al.*, 1968). All these effects are understandable in terms of the double helix model and we therefore propose that gel melting and setting proceed by processes shown in Figure 7,

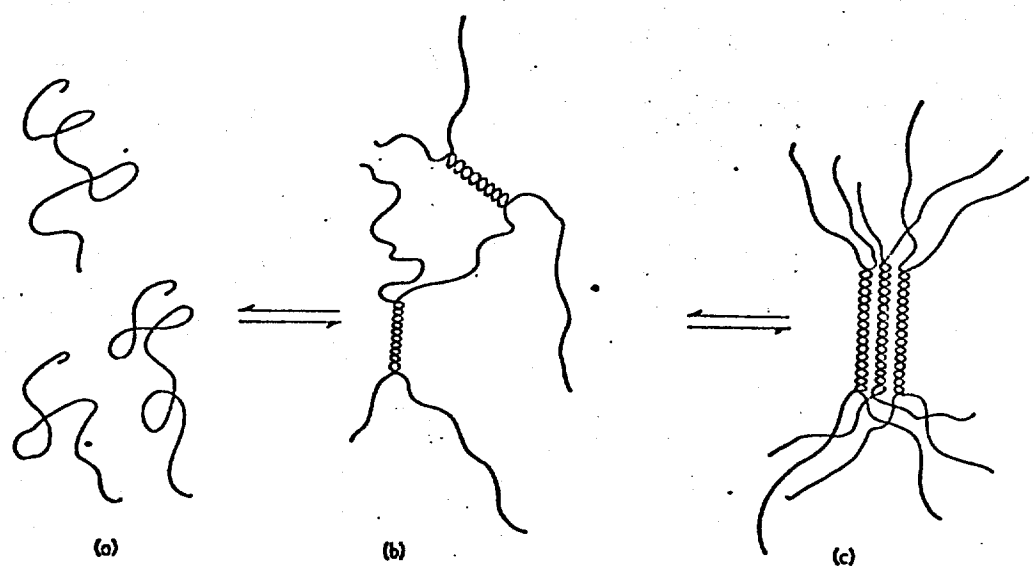


FIG. 7. Suggested relation of polysaccharide chains (a) in solution, (b) and (c) in the gel. The extent to which the helices are aggregated in the gel is not yet known, but (c) must represent the oriented fibre.

and that biological texture is regulated by control of helix content *via* primary structure. Other polysaccharide sulphate networks, of animal and seaweed origin, have primary structures based on an alternating arrangement of 1,3 and 1,4 linkages. Model building suggests that their ranges of allowed conformations are similar to the carrageenans (Rees, 1969*a*) and possible relationships are now under experimental investigation.

We are grateful to Dr S. Arnott for advice and helpful discussions, to Mrs C. S. Banks for measuring fibre densities, to the Science Research Council for studentships to two of us

(J. W. C. and J. W. B. S.), to Marine Colloids, Inc., for a studentship to one of us (N. S. A.) and for polysaccharide samples, to the Edinburgh Regional Computing Centre for their co-operation, and to Professor Sir Edmund Hirst for his interest and encouragement.

APPENDIX

Model Building Calculations for Alternating Polysaccharides

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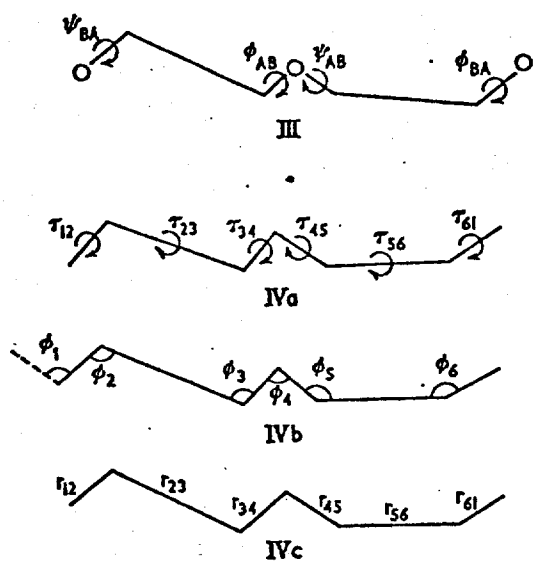
The following procedure ("Modelbuild II") can be applied to any alternating polysaccharide chain without 1,5 and 1,6 linkages, in any conformation. It is illustrated here by reference to carrageenans. The chain (II) is treated as a backbone (III) on which other groups are fixed appendages. Following the methods and notations of Sugeta & Miyazawa (1967), the helical parameters, n and h, can be expressed in terms of the angles of internal rotation τ_{ij} (see IVa), the angles between vectors and bonds ϕ_i (see IVb), and the interatomic distances r_{ij} (see IVc). All these quantities are implicit in the starting assumptions (Rees 1969*a*; and also p. 92) except τ_{12}, τ_{34}, τ_{45} and τ_{61} which correspond, respectively, to ψ_{BA}, ϕ_{AB}, ψ_{AB} and ϕ_{BA}. A rotation matrix, T, can also be derived for use in transferring the polysaccharide in any conformation to the helix axes defined by Sugeta & Miyazawa (1967).



The calculation proceeds in the stages described below; stages 1 to 6 were done together in one computer programme, stage 7 by hand, and stages 8 to 13 in a second programme.

(1) Co-ordinates of sugar residues A and B were obtained on axes placed at each O(1), as defined before (Rees, 1969*a*).

(2) A second set of co-ordinates was generated, for each residue, referred to axes at each non-reducing glycosidic oxygen with Ox along the corresponding O—C bond (O(3)—C(3) for B and O(4)—C(4) for A); each xy plane is defined by C(1) so that this atom has a positive y co-ordinate.

(3) The fixed parameters, τ_{ij}, ϕ_i, r_{ij}, were calculated or (for ϕ_1 and ϕ_4) assigned.

(4) Using the initial co-ordinates for A and the co-ordinates of B from stage 2, the combinations of τ_{34} and τ_{45} were obtained which are not excluded by outer limit criteria. Each angle was varied stepwise from 0° to 360° for every value of the other and the B co-ordinates were referred to the A axes at each stage, by successive rotation of the B axes about Ox through τ_{45}, about Oz through $\pi - \phi_4$ and then about Ox through τ_{34}; this allows all interatomic distances to be calculated so that sterically impossible conformations can be rejected.

(5) A list of all possible combinations of τ_{12} and τ_{61} was obtained similarly.

(6) Each allowed combination of τ_{34} and τ_{45} was taken with each allowed combination of τ_{12} and τ_{61}, and with the fixed values from stage 3, to calculate n and h.

(7) The results from stage 6 were plotted on $n - h$ graphs (Ramachandran *et al.*, 1963), holding two angles constant at a time; n and h were found to change smoothly with the torsion angles. The conformations required were at the intersections of the $n = \pm 3$ curves with the curve corresponding to the value of h derived from the diffraction photograph. Some recalculation around the intersection was usually required to locate these conformations accurately. The process was repeated for all six pairs of angles. The result is a list of sets of τ_{12}, τ_{34}, τ_{45}, and τ_{61} values which would give the measured or postulated n and h.

(8) For each τ_{ij} set from the previous stage, a disaccharide residue (B — A) in the corresponding linkage conformation (τ_{61}, τ_{12}) was generated on the B axes of stage 2. This residue was then rotated about Ox, in the appropriate sense, through τ_{45}. The result is the chemical repeating unit of the polysaccharide with respect to fixed molecular axes and in a conformation which corresponds to a particular τ_{ij} set.

(9) Each τ_{ij} set was used with the parameters from stage 3 to calculate the elements of the T matrix (Sugeta & Miyazawa, 1967) which rotates the molecular axes to make them parallel with the helix axes, and ρ, the distance between the origin of the molecular axes and the z axis of the helix.

(10) The results of the preceding stage were used to transfer the disaccharide repeating unit (stage 8) to the helix axes and hence, with n and h, to generate a complete turn of helix, or more.

The checking of contacts between non-adjacent residues and residues in different chains, and the placing of substituents such as sulphate esters, then follows as outlined in the main part of the paper.

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(10)

The anticoagulant activity of carrageenan

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λ-Carrageenans and κ-carrageenans from samples of *Chondrus crispus*, carrageenan from *Polyides rotundus* and degraded carrageenan from *Eucheuma spinosum* have anticoagulant activity on intravenous injection in the rabbit. Anticoagulant activity appears to be caused by a general reaction with plasma protein. The undegraded carrageenans are acutely toxic on intravenous injection and form insoluble complexes with fibrinogen in neutral solution. Degraded carrageenan is very much less toxic and, like heparin, forms soluble complexes with fibrinogen. The λ-carrageenans from *C. crispus* have higher sulphate content, consistently prolong the clotting time more, and are more toxic than the corresponding κ-carrageenans. The differences in sulphate content between the various λ-carrageenans, and between the carrageenans from the other seaweeds tested, do not correspond directly with differences in anticoagulant action and toxicity.

THE carrageenans, which occur naturally in the red seaweeds, constitute a closely related group of sulphated galactans. While displaying the biological properties of sulphated polysaccharides in general, quantitative differences exist between the members of the group and between the readily separated κ- and λ-carrageenans which are present in certain of the members. Houck, Morris & Lazaro (1957) examined unfractionated whole extracts of a number of seaweeds for anticoagulant activity which they found only in *Gigartina acicularis*. Hawkins & Leonard (1962, 1963) fractionated one *Chondrus crispus* carrageenan into its κ- and λ-components and found greater anticoagulant activity in the λ-carrageenan which contained more ester sulphate than the κ-carrageenan. Rees (1963) has suggested that λ-carrageenan may be the biological precursor of κ-carrageenan. Whole extracts of seaweeds might therefore be expected to vary in the relative content of κ- and λ-carrageenans and hence in anticoagulant activity. This may explain largely negative findings such as those of Houck & others (1957).

We report a study of the anticoagulant activity of a group of carrageenans differing in ester sulphate content, molecular weight and source.

Materials and methods

Animals. Male New Zealand white rabbits (2–4 kg) were used. Food, but not water, was withheld for 18 hr before testing. Each animal acted as its own control and none was used more than once.

Collection of blood. Blood was allowed to drip freely into Pyrex glass tubes from a small incision made on the marginal vein of the shaved, warmed and solvent-cleaned ear. The first ml of blood was discarded.

All clotting tests were made at 37°. Control results were obtained from blood withdrawn immediately before injection of carrageenan; test bloods were withdrawn 2 hr after the injection of carrageenan, or 0·5 hr in the heparin experiments. Preliminary experiments showed that the greatest anticoagulant effect of the carrageenans occurred 2 hr after injection while that for heparin occurred at 0·5 hr.

Saline. 0·85% w/v sodium chloride in water for injection, B.P.

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Carrageenans. Degraded carrageenan (Ebimar, Evans Medical Ltd.). The other carrageenans, the κ- and λ-fractions (Black, Blakemore Colquhoun & Dewar, 1965), and the data in Table 1 were provided by the Arthur D. Little Research Institute through the courtesy of Dr. E. T. Dewar. The carrageenans were injected intravenously in 8 ml saline solution.

Heparin. Sodium heparin, 150·1 units/mg (Evans), kindly supplied by Dr. C. H. Smith.

Thrombin solution. Thrombin Topical (Maw) 20 u/ml in saline.

Citrated plasma. Blood (9 ml) was allowed to drip into trisodium citrate solution (1 ml; 3·13% w/v), mixed, and centrifuged at 1400 rpm for 10 min.

Fibrinogen. Prepared according to Biggs & Macfarlane (1962). A saline solution containing 1 mg/ml was used in the clotting tests.

Whole blood clotting time. The test of Lee & White (1913) in Pyrex glass tubes (10 × 75 mm) was used.

Thrombin time. Citrated plasma, (0·2 ml) and saline, (0·1 ml) were mixed and thrombin solution, (0·1 ml) was added. The time between addition of thrombin and solid clot formation was noted. Triplicate determinations were done on each plasma and results calculated as follows:

$$\frac{\text{average clotting time of control plasma}}{\text{average clotting time of test plasma}} \times 100$$

In the *in vitro* experiments (Table 3) saline (0·1 ml) in the control was replaced by carrageenan solution (0·1 ml) in the test and normal citrated rabbit plasma used.

One-stage and two-stage prothrombin tests; prothrombin consumption test. The methods of Biggs & Macfarlane (1962) were used and results were expressed as follows:

one-stage: $\frac{\text{average clotting time of control plasma}}{\text{average clotting time of test plasma}} \times 100$

(triplicate determinations on each plasma)

two-stage: $\frac{\text{area under test curve}}{\text{area under control curve}} \times 100$

prothrombin consumption index:

$$\frac{\text{minimum plasma clotting time}}{\text{minimum serum clotting time}} \times 100$$

In vitro prothrombin times (Table 3) were obtained using a one-stage test modified to include saline (0·1 ml) in the control and carrageenan solution (0·1 ml) in the test. 50% increase in time was taken as standard because certain carrageenans caused precipitation rather than clotting when used in excess of the amount required to prolong the clotting time by 50%.

Fibrinogen-carrageenan precipitation. Aqueous fibrinogen (1 ml; 0·4%) was mixed at room temperature with aqueous carrageenan (1 ml) of various concentrations. pH of the mixture was 6·8. Mixtures were allowed to stand 24 hr before reading against a fibrinogen solution as control.

Carrageenan was determined by the method of MacIntosh (1941).

Platelet counts. The method of Brecher & Cronkite (1950) was used.

Results and discussion

The results are in Tables 2–7. All results from *in vivo* experiments are means from four rabbits.

Anticoagulant action of the carrageenans. In the whole blood clotting test all the λ-carrageenans were, in varying degree, active at 3 mg/kg (Table 2) but toxicity began to appear at 5 mg/kg. Table 2 reveals a trend to greater activity with higher sulphate content (Table 1) amongst the four λ-carrageenans, but the carrageenan with the highest sulphate content did not have the greatest activity, *Polyides* carrageenan having greatest effect. It is nevertheless clear that, for the *Chondrus* carrageenans,

TABLE 1. SOURCES AND PROPERTIES OF THE CARRAGEENANS USED

Carrageenan	Code	Source	SO_4Na(%)		3,6-Anhydro galactose, (%)		Inherent viscosity (dl/g)	
			κ	λ	κ	λ	κ	λ
Chondrus crispus	–CY	Yarmouth, Nova Scotia	28·2	37·3	29·2	3·5	13·7	16·2
" "	–CNS	Northumberland Strait, Nova Scotia	28·4	34·9	25·3	4·1	20·8	21·7
" "	–CSE	Sebasco Estates, Nova Scotia	29·8	32·3	25·2	9·1	14·3	13·8
" "	–CMI	Mud Island, Nova Scotia	27·0	32·2	24·8	9·8	8·6	9·4
Polyides rotundus		Moose Head, Nova Scotia	35·0		2·3		5·1	
Degraded-λ from *C. crispus*-CNS			30·7		—		1·3	
Degraded carrageenan (*Eucheuma spinosum*)		S.E. Asia (Evans Medical Ltd.)	29·0		21·0		0·3	

Inherent viscosity, $\eta_{inh} = c^{-1}\ln(\eta_{soln.}/\eta_{solv.})$ dl/g where c = g solute in 100 ml solution, was measured at 25° in an Ostwald viscometer (M2 BS U/M) using 0·1 M sodium chloride as solvent. For undegraded carrageenans c = 0·02; for degraded carrageenans c = 0·2. Viscosity is taken as a comparative indication of molecular weights amongst these substances which all have similar structure.

TABLE 2. RABBIT WHOLE BLOOD CLOTTING TIMES 2 HR AFTER INTRAVENOUS CARRAGEENAN; LOWEST INTRAVENOUS DOSES OF CARRAGEENANS KILLING WITHIN 24 HR

Carrageenan	Clotting time, min. mg/kg i.v. 5		3		Lowest dose killing within 24 hr mg/kg i.v.	
	κ	λ	κ	λ	κ	λ
C. crispus –CY	11	14	6	10	3	1
" –CNS	8	toxic	9	13	5	1
" –CSE	7	8	6·5	7·5	10	5
" –CMI	6	10	5	9	15	5
Degraded λ–CNS		8		9	10	
P. rotundus		—		20	5	
Degraded carrageenan	200 mg/kg 17		100 mg/kg 10		>1000	
Heparin	(200 u/kg) 20		(75 u/kg) 8		—	

Results are averages from four rabbits.

TABLE 3. WEIGHTS OF CARRAGEENANS REQUIRED TO PROLONG THE PROTHROMBIN AND THROMBIN TIMES *in vitro* BY STATED AMOUNTS

Carrageenan	Weight required to prolong the prothrombin time by 50% µg		Weight required to prolong the thrombin time by 100% µg	
	κ	λ	κ	λ
C. crispus -CY	35	5	12	<1
" -CNS	35	3	7	1
" -CSE	35	12	8	2
" -CMI	45	15	30	2
P. rotundus	5		1	
Degraded carrageenan	200		50	

Results are averages from plasmas of four rabbits.

each λ-carrageenan has higher sulphate content and greater effect than its κ-counterpart. Although this confirms the belief that the λ-carrageenans are generally more active anticoagulants than the corresponding κ-carrageenans, the results for all the carrageenans, and also the high dose required for degraded carrageenan, suggest that anticoagulant activity does not depend only on ester sulphate content. This conclusion is supported by the results (Table 3) of *in vitro* experiments in which different stages in the clotting mechanism are examined.

Degraded carrageenan is a degraded κ-carrageenan; it has a high 3,6-anhydrogalactose content and the parent carrageenan extracted from *Eucheuma spinosum* can be degraded by mild mineral acid treatment without serious sulphate hydrolysis. This degradation results in a much smaller molecule, permits a higher dose unaccompanied by the toxicity of the undegraded κ- and λ-carrageenans, and anticoagulant activity can be clearly demonstrated. But with undegraded κ-carrageenan, even at the highest safe dose, anticoagulant activity was either low or absent. Amongst the undegraded κ-carrageenans, the most active (κ-CY) was the most toxic and, conversely, the least toxic (κ-CMI) showed the least activity; it also had the lowest viscosity. It is unlikely that the difference in sulphate content (1%) between these two, accounts for the difference in anticoagulant activity, a conclusion which is supported by the data (Tables 1, 2 and 3) for the other two κ-carrageenans. Discussing a related group of polysaccharide sulphates, the laminarin sulphates, Adams, Heathcote & Walker (1962) stated that the laminarin "with the highest molecular weight and greatest degree of sulphation has high anti-lipaemic and anticoagulant activity, and toxicity".

λ-Carrageenans have a low, and κ-carrageenans a high, 3,6-anhydrogalactose content, and this is one of the distinguishing features between the κ- and λ-carrageenans (Table 1). That the presence of 3,6-anhydrogalactose is not responsible for lower activity of the κ-carrageenans is shown by the anticoagulant activity of degraded carrageenan at adequate dosage.

Intravenous toxicity of carrageenans. Large doses of sulphated polysaccharides can be given orally to man and animals, little or no absorption occurs, and direct systemic toxicity by this route has never been observed.

TABLE 4. CARRAGEENAN-FIBRINOGEN REACTION IN WATER (pH 6·8)

Carrageenan	μg carrageenan mixed with 4 mg fibrinogen in 2 ml mixture								
	160	80	40	20	10	5	2·5	1·25	0·6
C. crispus–λ-CY	—	—	—	+	+	++	+++	+++	+++
" λ-CNS ..	—	—	—	0	0	++	+++	+++	+++
" λ-CSE	—	—	—	—	0	+	++	++	++
" λ-CMI ..	—	—	—	—	—	0	0	+	+
" κ-CY	—	—	0	+	+	+	+	+	+
" κ-CNS	0	0	0	+	+	+	+	+	+
" κ-CSE	—	—	—	—	—	0	+	+	+
" κ-CMI	—	—	—	—	—	0	0	0	+
P. rotundus	—	—	—	—	0	+	++	++	
Degraded carrageenan ..	—	—	—	—	—	—	—	—	—
Heparin	—	—	—	—	—	—	—	—	—

— = clear solution after mixing (see text); 0 = mixture has same opalescence as control (i.e. no evidence of complex formation); + = increased opalescence (finely dispersed precipitate); ++ = flocculated precipitate; +++ = copious flocculated precipitate.

Nevertheless, these substances are toxic when administered intravenously. Astrup (1953) and Walton (1954) found toxicity by this route in certain high molecular weight dextran sulphates and concluded that the toxic reactions were initiated by formation, in the circulation, of insoluble fibrinogen complexes which entrapped platelets and blood cells. The carrageenans can be grouped according to the nature of their reaction with fibrinogen at pH 6·8, which is above its isoelectric point. In distinction from the less active, less toxic, κ-carrageenans which formed small particle dispersions with fibrinogen, the λ-carrageenans formed coarser precipitates which were flocculent or coagulated. Both types of precipitate were soluble in excess of the respective carrageenan. Table 4 shows that insoluble complex formation was much more pronounced with the CY-carrageenans, and slightly more pronounced with the CNS-carrageenans, than with the other carrageenans. They were also the most active and toxic of the *Chondrus* group. The λ-carrageenans did not all form similar types of precipitate: thus λ-CY and λ-CNS formed fibrous floating coagula immediately, but λ-CSE and λ-CMI required up to 0·5 hr to form particulate precipitates. Assuming Walton's (1953) conclusions regarding the trapping of platelets by dextran sulphate-fibronogen precipitates to be relevant to the present case, the differences in character of the formed precipitates could be related to the different activities and toxicities of the λ-carrageenans. With the κ-carrageenans, on the other hand, there was little or no evidence to suggest a relationship between sulphate content, precipitation, and anticoagulant activity.

Maximum precipitation occurred for most carrageenans when 2·5 μg reacted with fibrinogen (4 mg; in 2 ml); greater concentrations of carrageenan resulted in less precipitation and eventually in soluble complex formation, indicated by the clearing of the opalescence contributed by the fibrinogen, and by results of estimation of free carrageenan in solution.

Soluble complex formation, seen at all concentrations with degraded carrageenan and heparin is associated in these two substances with relative absence of toxicity. Toxicity is associated with sulphated polysaccharides which can form insoluble complexes with fibrinogen at neutral pH.

The relationship of fibrinogen precipitation *in vitro* to toxicity and

activity *in vivo* is obscure. Concentrations in the precipitation experiments were chosen to simulate *in vivo* concentrations as far as was possible; thus a carrageenan dose of 3 mg/kg, given intravenously to a rabbit having

TABLE 5. PERCENTAGE REDUCTION OF PLATELETS IN THE RABBIT 2 HR AFTER INTRAVENOUS CARRAGEENANS

Carrageenan	Dose mg/kg i.v.	Percentage reduction in platelet count
C. crispus κ-CY	5	24
" κ-CNS	5	39
" κ-CSE	5	25
" κ-CMI	5	0
" λ-CY	5	62
" λ-CNS	5	59
" λ-CSE	5	42
" λ-CMI	5	31
P. rotundus	3	42
Degraded carrageenan	200	32
Heparin	200 u/kg	0

Results are averages from four rabbits.

a blood volume of 200 ml, would, if completely mixed with the blood, give 90 μg carrageenan in 2 ml blood. It was below this concentration that precipitation *in vitro* began, and maximum precipitation was only seen when the carrageenans were even more dilute (Table 3).

In similar experiments thrombin-carrageenan complexes also precipitated, but with thrombin, as distinct from fibrinogen, heparin and degraded carrageenan formed insoluble precipitates at appropriate concentrations. Carrageenan affected all the tests of clotting examined (Tables 2, 6, 7) and this suggests that their reaction with plasma proteins, especially those concerned with clotting, is a general one and not restricted to fibrinogen; it is possible that insoluble complex formation with other proteins, whether engaged in the clotting or not, could also contribute to toxicity by embolism formation. Complexes formed with clotting proteins need not, on the other hand, be insoluble to affect the clotting reaction.

Platelet counting showed (Table 5) that injection of λ-carrageenans resulted in lower platelet counts than the injection of κ-carrageenans, and amongst the λ-carrageenans the most active anticoagulants caused greatest reduction in count. Although such a trend was obscure amongst the κ-carrageenans, it is noteworthy that κ-CMI caused no reduction in platelet count and was the least active and the least toxic of the κ-carrageenans. Heparin and degraded carrageenan, both relatively non-toxic, caused little or no reduction in platelet count. When the platelet counts were very low, agglutinated platelets were never seen, but with normal or slightly reduced counts (as seen with the κ-carrageenans) agglutination was occasionally seen. It is appropriate to speculate that the structure of the κ-carrageenan-plasma protein complex is unable to trap platelets which are nevertheless caused to agglutinate by the κ-carrageenans. The structure of the λ-carrageenan-plasma protein complex is able to trap the agglutinated platelets, resulting in depleted numbers of unagglutinated platelets in the circulation. Such differences

in structure are more likely to be due to difference in configuration of the sulphated polysaccharides rather than to relatively small differences in sulphate content and molecular weight. This view is supported by the fact that degraded λ-carrageenan is much more toxic than degraded

TABLE 6. EFFECTS OF INTRAVENOUS CARRAGEENANS ON THROMBIN AND PROTHROMBIN TESTS IN THE RABBIT

Carrageenan	Thrombin time				One-stage prothrombin				Two-stage prothrombin			
	mg/kg i.v.				mg/kg i.v.				mg/kg i.v.			
	5		3		5		3		5		3	
	κ	λ	κ	λ	κ	λ	κ	λ	κ	λ	κ	λ
C. crispus –CY	83	68	91	76	78	58	95	69	55	38	61	50
" –CNS	85	toxic	85	87	84	toxic	81	49	63	toxic	56	36
" –CSE	87	55	82	74	79	62	78	75	66	57	59	59
" –CMI	102	67	97	94	92	63	87	70	71	68	82	56
P. rotundus			72				58				43	
Degraded carrageenan 200 mg/kg	60				69				17			
Heparin												
200 u/kg	<1				86				9			
75 u/kg	8				100				24			

κ-carrageenan even though both have similar sulphate content and only a small difference in viscosity. The different effect on platelets could also be causally concerned in the different anticoagulant activities of the κ- and λ-carrageenan, although this suggestion is, with present knowledge, not amenable to searching experimental examination.

The general pattern of the fibrinogen reaction of the carrageenans therefore resembles that of certain dextran sulphates, and the picture of respiratory and circulatory collapse with generalised congestion of abdominal organs, in rabbits given a fatal dose, conforms to the description of dextran sulphate overdosage described by Walton (1954) and by others who have observed the toxic effects of parenterally-administered macromolecules.

Localisation of anticoagulant action. The reaction of the carrageenans with plasma protein can explain their toxicity; fibrinogen depletion, caused by its reaction with carrageenan, could affect the final clotting stage. However, the rapid removal of some fibrinogen from the circulation, cannot wholly account for the anticoagulant action which was not fully developed in the rabbit for any of the carrageenans until 2 hr after injection, whereas that of heparin was present at 0·5 hr.

Hawkins & Leonard (1962) deduced that the anticoagulant action of carrageenan was antithrombic in nature on the basis that thrombin time increased with increasing plasma concentration of carrageenan; in agreement with the same authors (1963), Table 3 shows that more carrageenan was required to prolong prothrombin than thrombin time *in vitro* but no quantitative relationship can be obtained from this comparison. Antithrombin action could obviously affect all clotting tests but this does not appear to be the sole mode of action. Thrombin time (Table 6)

was only slightly affected by the carrageenans as compared with heparin which gave a marked effect even at the low dose. This is in contrast to the

TABLE 7. PROTHROMBIN CONSUMPTION INDEX BEFORE AND AFTER INTRAVENOUS INJECTION OF CARRAGEENANS

	mg/kg i.v.							
	5				3			
	κ		λ		κ		λ	
Carrageenan	before	after	before	after	before	after	before	after
C. crispus -CY	6	31	11	42	11	30	5	49
" -CNS ..	7	46	toxic		9	72	6	52
" -CSE ..	5	42	24	115	13	22	4	29
" -CMI ..	3	19	7	66	9	29	14	56
P. rotundus					before 13		after 146	
	200 mg/kg							
Degraded carrageenan	before 11				after 105			
	200 u/kg				75 u/kg			
Heparin	before 5		after 335		before 5		after 5	

similarity in effect in the whole blood clotting time, between *Chondrus* carrageenans and the low dose of heparin, and between degraded carrageenan and the high dose of heparin. This difference between carrageenan and heparin suggests that carrageenan may affect the earlier stages of clotting which precede prothrombin conversion. These earlier stages are associated with prothrombin activator (thromboplastin) formation which can be followed by the prothrombin consumption test. Reduced prothrombin consumption (Table 7) is compatible with the actions of carrageenan already shown, namely platelet count reduction, and effects on fibrinogen and probably on other clotting factors.

The results of the one-stage and two-stage tests (Table 6) support the suggestion that carrageenans interfere with clotting factors in a non-specific manner.

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(7)

Degraded and undegraded carrageenans and antipeptic activity

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The antipeptic activities of a series of undegraded κ- and λ-carrageenans and degraded carrageenans have been examined by two methods. The activities differ quantitatively. Compared with the κ-carrageenans, the λ-carrageenans, as a group, have greater activity and this is associated with low 3,6-anhydro-D-galactose and high ester sulphate content. Within the series of undegraded carrageenans, both κ and λ, differences in antipeptic activity are significantly and positively related to differences in sulphate content. In addition to the effect of sulphate, 3,6-anhydrogalactose content appears to be inversely related to activity but in the λ- series only; differences in viscosity are without effect. Initial degradation without loss of sulphate resulted in loss of activity but further degradation, also without loss of sulphate did not reduce activity further. Antipeptic activity appears to depend on a combination of structural features of which the differences between κ-λ carrageenans, molecular size and polyanionic properties are aspects.

THE addition of sulphated polysaccharides to gastric juice or to solutions of pepsin results in a decrease in demonstrable proteolytic activity. This has been stated to be due to complex formation between the sulphated polysaccharide and the enzyme in the instance of chondroitin sulphate (Levey & Sheinfeld, 1954). For degraded carrageenan (Anderson, 1961), protection of the substrate rather than direct anti-enzyme action was shown to be principally responsible for the decrease in peptic activity.

Because of the interaction between sulphated polysaccharides and protein (substrate or enzyme, or both) at appropriate pH, which depends on the polyionic nature of the substances concerned, it has been inferred that ester sulphate is the part of the carrageenan molecule responsible for the magnitude of antipeptic activity. Indeed, it has been shown that for certain sulphated polysaccharides, sulphate content can be related to activity (Hawkins & Leonard, 1962; Ravin, Baldinus & Mazur, 1962). However, comparison of published work suggests that sulphate content is not the only part of the molecule concerned in antipeptic activity. The relationships are even less clear where different methods of measuring peptic activity have been used.

In the present work, the relationship between antipeptic activity and sulphate content, and other aspects of carrageenan structure, is examined using two methods of measuring antipeptic activity.

Experimental

MATERIALS AND METHODS

Carrageenans. Undegraded and degraded carrageenans were used, and were obtained from the principal seaweed sources, *Chondrus*, *Gigartina* and *Eucheuma* species. Of the undegraded carrageenans, unfractionated samples and samples fractionated into κ- and λ-components were used. With the exception of a degraded carrageenan from *Eucheuma spinosum*—C16—(Anderson, 1961), and Rees' carrageenan (Rees, 1963) kindly

From the Department of Pharmacy, University of Strathclyde, Glasgow.

supplied by Dr. D. A. Rees, Chemistry Department, University of Edinburgh), all other carageenans (Black, Blakemore & others 1965) and the data in Table 1 were provided by the Arthur D. Little Research Institute, through the courtesy of Dr. E. T. Dewar.

Gastric juice. Gastric juice was obtained from duodenal ulcer patients undergoing the augmented histamine test and was used fresh. (It was provided through the courtesy of Dr. A. I. M. Glen, Western Infirmary, Glasgow.) Insoluble material was removed by centrifugation. Occasional juices devoid of acidity or discoloured were not used.

PEPTIC ACTIVITY

Method A was a modification of the method of Anson (1938). Two concentrations of carrageenan were used: 20 or 80 mg carrageenan was dissolved in 2 ml hydrochloric acid solution (pH 1·6) and this was added to 2 ml pepsin solution (20 mg/100 ml; pH 1·6; Armour crystalline porcine pepsin) giving concentrations of 0·5 and 2% of carrageenan respectively in the 4 ml of acid-pepsin at pH 1·6. This solution was incubated at 37° for 10 min and 2 ml haemoglobin solution (1·2%; pH 1·6; Armour bovine haemoglobin for proteolytic enzyme assay) was added, followed by incubation at 37° for 30 min. After incubation, 20 ml of 10% trichloroacetic acid was added, the flasks allowed to stand in the water bath for 1 hr and the contents filtered (Whatman No. 3). 5 ml filtrate was added to 10 ml N sodium hydroxide followed by 3 ml of a 1:2 aqueous dilution of Folin-Ciocalteu reagent. The extinction developing in 10 min was read at 660 mμ. Appropriate blanks and controls were included.

Method B was a modification of the method of Hunt (1948). Three concentrations of carrageenan were used: 8, 12 or 20 mg carrageenan was dissolved in 2 ml hydrochloric acid solution (pH 2·1) and 2 ml of centrifuged supernatant of the gastric juice added. (8 mg carrageenan in this 4 ml volume gave 0·2%.) 1 ml of this solution was used in the test for peptic activity. Appropriate controls and blanks were included. The remainder of the method was as described by Hunt. Extinction values were converted to Hunt units.

Antipeptic activity. This term was calculated from $(1 - I/S) \times 100$ where I and S represent peptic activity with and without inhibitor respectively, corrected for blanks as necessary. Antipeptic activity is therefore the amount of inhibition, %.

Results and discussion

A representative group of carrageenans from species of the red seaweed families *Chondrus*, *Gigartina*, *Eucheuma* and *Polyides* has been examined for antipeptic activity by two methods which differ both in enzyme and substrate. Method A uses a purified enzyme, whilst method B uses fresh human gastric juice from duodenal ulcer patients. Two methods were used to increase the likelihood of finding a pattern of antipeptic activity amongst the carrageenans and, hence, not only a basis for a standard method of assessing their antipeptic activity, but also information

about their mode of action. These two methods have been used in, or have formed the basis of, most reports on antipeptic activity.

The carrageenans were selected from a number of such substances which became available during an extensive survey (Black & others, 1965) of carrageenan as a possible therapeutic agent for the treatment of peptic ulcer. κ- and λ-Components were studied because they differ in structure and properties (Rees, 1966). Undegraded and degraded carrageenans were examined because, although degradation usually reduces antipeptic activity, it is possible in therapy and in pharmacological investigation to use greater amounts of the less viscous, more rapidly soluble degraded product, thus obtaining greater activity and more information than would otherwise be possible with the highly viscous, slowly soluble, native

TABLE 1. SOURCES AND PROPERTIES OF THE CARRAGEENANS USED

	Code	SO_3Na (%)		η_{inh} (dl/g)		3,6-anhydro-D-galactose $C_6H_8O_4$ (%)	
		κ	λ	κ	λ	κ	λ
Undegraded							
Chondrus crispus	CMI[1]	27·6	32·2	8·6	9·4	24·8	9·8
" "	CSE[2]	29·8	32·3	14·3	13·8	25·2	9·1
" "	CBC[3]	29·6	34·6	11·6	14·4	22·9	4·7
" "	CNS[4]	28·4	34·9	20·8	21·7	25·3	4·1
" "	CY[5]	28·2	37·3	13·7	16·2	22·2	3·6
" "	CRF[6]	—	38·8	—	15·1	—	4·2
" "	CCB[7]	—	47·6	—	13·1	—	4·6
	CNB[8]	43·6 (unfractionated)				18·8	
Rees' carrageenan	R-λ[9]	—	41·1	—	23·2	—	1·5
Gigartina stellata	GS[10]	30·1	28·3	15·0	13·6	22·6	15·9
G. radula	GR[11]	31·0	35·6	15·7	15·7	23·2	9·6
G. pistillata	GP[12]	35·0	43·6	10·5	20·6	12·8	4·2
Eucheuma spinosum	ES[13]	37·7		7·2		19·0	
Polyides rotundus	PR[14]	36·3		5·1		2·3	
Degraded							
(*E. spinosum*)	C16[15]	36·1		0·3		21·0	
C. crispus	CY-λ-D1[16]	30·5		6·4			
" "	"-D2	29·7		3·0			
" "	"-D3	33·5		1·2			
" "	"-D4	38·2		3·8			
" "	"-D5	39·2		2·3			
" "	"-D6	37·3		1·35			
" "	"-D7	38·2		1·04			
" "	"-D8	37·2		0·66			
" "	"-D9	39·2		0·45			
" "	"-D10	39·2		2·80			
" "	"-D11	39·4		1·72			
G. pistillata	GP-λ-D2	37·7		2·6			
" "	"-D3	42·5		2·23			
" "	"-D4	40·6		1·58			
" "	"-D5	40·9		1·04			
" "	"-D6	40·9		0·72			
" "	"-D7	40·1		0·65			
E. spinosum	ED3	37·2		1·37			
" "	" 4	35·8		0·94			
" "	" 5	37·5		0·68			
" "	" 6	37·7		0·54			

Inherent viscosity (logarithmic viscosity number), $\eta_{inh} = c^{-1}\ln(\eta_{rel}/\eta_{solvent})$ dl/g where c = g solute in 100 ml solution, was measured at 25° in an Ostwald viscometer (M2 BSU M) using 0·1M sodium chloride as solvent, for undegraded carrageenans, c = 0·02; for degraded carrageenans, c = 0·2.

Source: [1]Mill Island, Nova Scotia; [2]Sebasco Estates, Maine, U.S.A.; [3]Co. Claire, Eire; [4]Northumberland Str., Nova Scotia; [5]Yarmouth, Nova Scotia; [6]Roscoff, Finistere, France; [7]Casco Bay, Maine, U.S.A.; [8]North Brittany, France; [9]Dr. D. A. Rees, University of Edinburgh (Rees, 1963); [10]Millport, Scotland; [11]South Africa; [12]Povoa de Varzim, Portugal; [13]S. E. Asia; [14]Moosehead, Nova Scotia; [15]Anderson (1961); [16]A. D. Little Research Institute (all this series = GP and ED series).

TABLE 2. ANTIPEPTIC ACTIVITY OF UNDERGRADED AND DEGRADED CARRAGEENANS

Carageenan	Method A				Method B			
	carrageenan concentration (%)				carrageenan concentration (%)			
	0·5		2		0·2		0·3	
	κ	λ	κ	λ	κ	λ	κ	λ
Undegraded								
CM1	10	33	40	62	10	23	17	29
CSE	26	37	32	57	28	12	27	38
CBC	29	67	69	80	13	52	40	100
CNS	16	62	76	79	20	81	100	100
CY	23	74	36	79	27	57	14	100
CRF	—	—	—			36	—	100
CCB	—	53	—	72	—	17	—	84
CNB	56		81		12		94	
R-λ	—	67	—	70	—	100	—	100
GS	20	29	69	61	7	8	100	100
GR	17	25	63	69	6	24	34	94
GP	39	78	28	86	18	100	57	100
ES	35		71		21		90	
PR	60		62		23		37	
Degraded								
C16	41				11[1*]			
CY-λ-D1					27			
″-D2					25	[2]		
″-D3					55			
″-D4					53			
″-D5	39				7			
″-D6	28				4			
″-D7	46				9			
″-D8	39				7	[3]		
″-D9	41				8			
″-D10	37				14			
″-D11	43				8			
GP-λ-D2	51				12[2]			
″-D3	43				15			
″-D4	45				14			
″-D5	46				11	[2]		
″-D6	49				13			
″-D7	45				8			
ED3	34				21			
″ 4	32				21	[3]		
″ 5	40				15			
″ 6	36				13			

All values are the mean of 2 results. Most undegraded carrageenans gave 100% inhibition at 0·5% in method B.
Concentrations are explained in the text.
[1] Method of degradation.
[2] Dilute HCl; [3] periodate; [4] hypochlorite; [5] H^+ ion-exchange).

material. Degradation was carried out (Black & others, 1965) with the retention of sulphate as a principal objective, because it is generally believed that the basis of the activity of these substances lies principally in the sulphate content. Also, a degraded κ-carrageenan from *E. spinosum* which combines low viscosity and high sulphate content has been shown to have promising therapeutic effect (Bonfils & Lambling, 1960; Demole, 1963; Evans, Nowell & Thomas, 1965). Degradation with sulphate retention also permits the effect of variation of molecular weight with constant polyanionic properties to be observed. The results are in Tables 1 and 2.

UNDEGRADED CARRAGEENANS

The λ-carrageenans (with the exception of λ-carrageenan from *Gigartina stellata*, GS-λ) have higher sulphate and lower 3,6-anhydrogalactose

content than the corresponding κ-carrageenans, whilst the logarithmic viscosity number, η_{inh}, is, with the exception of carrageenan from *G. pistillata*, broadly similar for corresponding κ-and λ-components (Table 1). *Eucheuma*, a naturally occurring κ-like carrageenan, and *Polyides* carrageenan, which differs in structural details from the κ- and λ-carrageenans from other species of seaweed (Black & others, 1965) and therefore appears unique, have relatively low viscosities.

Analysis of activities. The antipeptic activities of the pairs of κ- and λ-carrageenans from eight samples of seaweed (5 C. *crispus*, 3 *Gigartina* species) were analysed separately. The 64 pairs of results were considered as a doubly replicated $2^3 \times 8$ factorial experiment, yielding Table 3.

TABLE 3. ANALYSIS OF VARIANCE FOR THE ANTIPEPTIC ACTIVITY OF THE κ- AND λ-CARRAGEENANS FROM FIVE CHONDRUS AND THREE GIGARTINA SEAWEEDS

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F-ratio
M	42·8	1	42·8	
C	29,585·3	1	29,585·3	
F	24,255·0	1	24,255·0	302·7
S	21,539·0	7	3,077·0	
MC	780·1	1	780·1	
MF	420·51	1	420·51	5·25
MS	4,106·7	7	586·7	
CF	21·13	1	21·13	0·26
CS	12,294·2	7	1,756·3	
FS	11,683·0	7	1,583·3	
MCF	442·5	1	442·5	
MCS	3,095·9	7	442·3	
MFS	2,183·0	7	311·9	
CFS	4,128·4	7	589·8	
MCFS	2,906·5	7	415·2	
Total = treatments	(116,584)	(63)		
Error	5,128	64	80·125	
Total	122,012	127		

M corresponds to the method factor (2 levels); C to the concentration factor (2 levels); F to the fraction factor (2 levels); S to the seaweed factor (8 levels).
0·1% point for F (1,64) ≐ 11·54 and for F (7,64) ≐ 7·07; 1% point for F (1,64) ≐ 7·07 and for F (7,64) ≐ 2·94; 5% point for F (1,64) ≐ 4·00 and for F (7,64) ≐ 2·17.

Study of this Table and the percentage points of the F-distribution with the appropriate degrees of freedom shows that of the 15 treatment combinations, all but two, namely the effect of the method used and the interaction of concentration with fraction (i.e. κ or λ), will have significantly large F-ratio values. This may be attributed to the relatively small error sum of squares obtained by measuring the variation of the results within each of the 64 cells. Of these 64 cells, 18 contained an equal pair of results and this would reduce the error sum of squares considerably. Although there is no difference between the results of the two methods, it has to be remembered that different concentrations of carrageenan have been used in the two methods. The conclusions drawn are: that despite the size of the error sum of squares there is a highly significant difference in the effects of the two fractions, κ and λ; that there is no interaction between the concentration and the fraction used, i.e. the effect of the different fractions is the same regardless of concentration; and finally, that the interaction of method used and fraction studied is slightly significant. Since the antipeptic activity of undegraded carrageenan is probably

mediated by substrate occlusion (as for degraded carrageenan), this could indicate differences in structure of the complexes formed between the κ- and λ-carrageenans and the proteins used in the methods.

Again, considering only the eight pairs of undegraded carrageenans, a multiple regression analysis was performed by computer, treating 3,6-anhydrogalactose (g), sulphate (s) and viscosity (v) as independent variables, and the logarithm of antipeptic activity (y) as dependent variable (both methods, 3 concentrations) for both κ- and λ-carrageenans. The antipeptic activities of the higher concentration in method B were omitted from the calculation because the proportionately large number of duplicate activities of 100 would tend to blunt the perception of the analysis. The regressions of y on s, y on s and v, y on s and g and y on g, s and v were studied separately for both κ- and λ-carrageenans.

Sulphate content. The regressions of antipeptic activity on sulphate were very highly significant (level of significance lower than 0·1 of 1%): differences in sulphate content determined differences in antipeptic activity amongst both κ- and λ-carrageenans in both methods at all concentrations studied, high sulphate content being associated with high activity. Differences in viscosity made no significant contribution to this conclusion.

3,6-*Anhydrogalactose content.* Higher 3,6-anhydrogalactose content, on the other hand, had significant association with lower effect (after due allowance for the effect of sulphate) only in the λ-carrageenans in method B and at the higher concentration in method A. With κ-carrageenans, where 3,6-anhydrogalactose content is higher than in the more active λ-carrageenan (t test showed significance at 0·1 of 1%), differences in 3,6-anhydrogalactose content did not contribute to differences in activity independently of sulphate.

Further interpretations of differences in activity require more knowledge of carrageenan structure and the nature of the interaction with substrate protein on which inhibition depends.

Molecular size. An apparent absence of effect of molecular size (as indicated by viscosity) is almost certainly due to the similarity which exists in this series of undegraded carrageenans.

Unfractionated extract. The effect of the κ-component in an unfractionated extract cannot yet be discerned, but the antipeptic activity of CNB carrageenan (Table 1), for example, does not match the high sulphate content. That this might be due solely to the presence of κ- carrageenan in the unfractionated extract tends to be discounted by the antipeptic activity of CRF-λ and CCB-λ where high sulphate content, especially in CCB-λ, is not associated with the highest antipeptic activity. There would therefore be unexplained exceptions to a "sulphate rule." It is not likely that incomplete fractionation of the κ- and λ-components could account for the exceptions which probably have their origins in other structural differences. κ- and λ-Carrageenans [and the "third component" (Rees, 1966)], are stages in a biosynthetic process and cannot, therefore, under conditions of growth and harvest, be expected to be at all times identical.

Antipeptic activity. The effect on antipeptic activity of the method used to determine it is seen in the individual cases of R-λ and GP-λ (Table 2). The high activity at lower concentration in method B for these carrageenans was not reproduced in method A, suggesting that these carrageenans may have some particular affinity for the enzyme or substrate in method B. With these carrageenans, and also with CY-λ and CNS-λ to some extent, it was observed that when they were added to gastric juice, insoluble globule-like structures were formed and it was usually when this occurred that extinction of peptic activity occurred. 100% antipeptic activity was not observed in the absence of this occurrence. Differences were also observed in the size of the floccules formed in the reaction between the carrageenans and the substrate, large floccules tending to produce greater inhibition than small ones. Attention has been drawn to a similar occurrence in the reaction between fibrinogen and carrageenans (Anderson & Duncan, 1965). Molecular size probably influences antipeptic activity by determining such differences in the nature of the reaction with substrate protein. R-λ and GP-λ, together with CNS-λ and CY-λ, have the highest viscosities of the series (Table 1). This suggests that there may be an optimum association of sulphate content and molecular properties for activity.

The antipeptic activity of *Polyides* carrageenan with its unique structure (Black & others, 1965) shows that activity is not monopolized by the κ-λ carrageenans. The activity of *Eucheuma* carrageenan, which occurs naturally without a λ-component, indicates that, with adequate sulphate the κ- configuration and a high 3,6-anhydrogalactose content do not preclude marked antipeptic activity.

DEGRADED CARRAGEENANS

Undegraded carrageenans generally dissolve slowly and to a limited extent in water to give solutions of very high viscosity. This raises practical problems in the study of the actions and uses of carrageenans. The anti-ulcer action of carrageenan follows inhibition of peptic activity (Anderson, 1961), mucosal coating (Anderson & Watt, 1959) and anti-secretory activity (Anderson, Marcus & Watt, 1962), and for these actions rapid dissolution after administration is desirable. Reduction of molecular weight with retention of activity would therefore be desirable to increase solubility. Of the λ-carrageenans, GP-λ and CY-λ were chosen, on the grounds of activity and availability, for degradative study in which sulphate content was left intact as far as possible. *Eucheuma* carrageenan was also studied because of its relatively high sulphate content and naturally low viscosity (Table 1), and also because of its susceptibility to degradation under mild acid conditions (British Patent, 840,623) explained by Black & others (1965) as being possible on account of the presence of the acid-labile 3,6-anhydrogalactose in the molecule.

Results for $\eta_{sp/c}$ (Table 1) show that much degradation could be effected in the series without serious loss of sulphate. However, with the exception of CY-λ-D3 and CY-λ-D4 (and to some extent CY-λ-D1 and CY-λ-D2) antipeptic activity was reduced as a result of degradation.

especially when measured by method B; it was also reduced, but to a lesser extent, in method A. Whether the retention of activity by CY-λ-D3 and -D4 is due to periodate degradation as opposed to hypochlorite degradation used for the others, is not known, but the antipeptic activity of GP-λ-D2, also periodate degraded, was about half that of CY-λ-D3 or -D4. CY-λ-D3 and -D4 have retained more sulphate than CY-λ-D1 or -D2. However, if this were the only factor CY-λ-D5 to -D9 might have been expected to be more active.

Degraded carrageenans were also examined at the higher concentrations in both methods, but the activities were either similar to, or less than, those at the lower concentrations. It is suspected that, at the higher concentrations, the degraded carrageenans may be forming trichloroacetic acid-soluble complexes with the substrate protein, with consequent interference with the estimation of the split products of the proteolysis. These results have therefore been omitted.

Otherwise adequate sulphate content turns out to be insufficient for high activity when certain λ-carrageenans are degraded by hypochlorite, and differences shown by the two methods of assessing activity are rather marked, suggesting that structures other than the sulphate are concerned in interaction with the different proteins in the two methods.

The analysis of the antipeptic activities of the undegraded carrageenans showed that such differences as existed in viscosity did not contribute to activity differences. However the reduction in viscosity effected by degradation, in the presence of substantially unaltered sulphate, suggests that marked changes in molecular size do affect antipeptic activity. The evidence therefore suggests that antipeptic activity is a function of the whole molecule, sulphate content and molecular size being principal features.

In use it may well be advantageous to employ degraded carrageenan the lower viscosity of which enables greater dosage to be used and faster dissolution to be achieved with more rapid distribution over the mucosa

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The absorption of carrageenans

SIR,—An understanding of the manner of gastrointestinal absorption of carrageenans would be useful in two ways. First, it would add to knowledge of the absorption of sulphated polysaccharides, including heparin, which is present on both sides of the gastrointestinal wall, but appears not to be absorbed in active form or quantity. Second, it would help to explain the actions of carrageenan in reducing histamine-induced parietal cell hyperplasia (Anderson & Soman, 1965a), in reducing secretion (Anderson, Marcus & Watt, 1962), and in preventing histamine-induced gastric ulceration in the pylorus-ligated guinea-pig when administered intraduodenally (Anderson & Soman, 1963). The last-named action indicated for the first time that the gastric effects of carrageenans (principally, anti-ulcer and anti-secretory) could be accomplished either after absorption or by one of several possible humoral effects (Giertz, Hähn, Schmutzler & Sesske, 1964; Anderson & Soman, 1966).

Carrageenans. Degraded κ-carrageenan from *Eucheuma spinosum* (C 16), degraded λ-carrageenan from *Gigartina pistillata* (GP-λ-D2), and undegraded λ-carrageenans from *Chondrus crispus* and *G. Pistillata* (CY-λ and GP-λ respectively) were used. Weight-average molecular weights (light-scattering) for the degraded carrageenans are 20,000—30,000; for the undegraded carrageenans they are 600,000—1,000,000.

Administration of carrageenans. Single intravenous injections of saline solutions of C 16 were given to male guinea-pigs, average weight 400 g, of P strain (Anderson & Soman, 1966) food, but not water, was withheld for 18 hr and at the end of this period urine was manually expelled from the bladder (or removed immediately after killing) and examined for sulphated polysaccharide. Qualitative examination was by staining with toluidine blue on filter paper; quantitative examination was by MacIntosh's (1941) method, suitably modified.

Oral administration was by inclusion in the drinking water, the initial and residual volumes over 18 hr being recorded. Food was withheld during the period. Oral consumption was also measured during experiments designed to demonstrate the anti-duodenal ulcer activity of carrageenan. Duodenal ulceration was produced by histamine acid phosphate (10 mg/kg in a wax-oil base, intramuscularly) (Anderson & Soman, 1965b).

Intraduodenal administration in 2 ml of saline was effected during anaesthesia (pentobarbitone sodium, 30 mg/kg, intraperitoneally) in the pylorus-ligated guinea-pig in an experiment designed (Anderson & Soman, 1963), to demonstrate the anti-gastric ulcer effect of carrageenan.

The results of intravenous injection of C16 are in Table 1. Increase in dose of

TABLE 1. URINARY CONTENT OF DEGRADED CARRAGEENAN AFTER A SINGLE INTRAVENOUS INJECTION TO GUINEA-PIGS

Group	Number of animals	Dose of degraded carrageenan mg/kg i.v.	Degraded carrageenan in urine mg/ml
1	4	2·5	0 (but present qualitatively)
2	3	4	0·03
3	4	5	0·05
4	3	5	0·03
5	4	7·5	0·13
6	5	7·5	0·11
7	3	10	0·07
8	3	12·5	0·09
9	3	15	0·33

C16 corresponds roughly to increase in urinary content of the polysaccharide. Assuming that it is degraded carrageenan which appears in the urine, the results show that between 2·5 and 5 mg/kg C16 given intravenously to the guinea-pig results in the appearance of urinary degraded carrageenan, indicating that there is no total renal barrier to its excretion. A method depending on metachromasia indicates that the polyanionic nature of the carrageenan is unchanged during its passage through the animal body.

C16 was also administered orally to guinea-pigs in drinking water (1%), over a period of 18 hr. When 293 mg of degraded carrageenan was consumed, the urine showed the qualitative presence of C16 only; using a 5% solution (1850 mg consumed) the urine contained about 0·3 mg/ml at the end of the experiment. Intraduodenal administration of 400 mg C16 in an anti-gastric ulcer experiment in the pylorus-ligated guinea-pig gave detectable, but not measurable, urinary metachromasia, indicating intestinal absorption. Gastric ulceration was reduced by 60%. GP-λ-D2 gave similar results. The experiment with GP-λ-D2 was conducted in association with an anti-duodenal ulcer test of this substance and the consumption of 675 mg of GP-λ-D2 was accompanied by 73% reduction in duodenal ulceration and demonstrable, but not measurable, urinary metachromasia. In an anti-gastric ulcer experiment the intraduodenal administration of 150 mg of GP-λ-D2 gave no demonstrable urinary metachromasia, but resulted in 47% reduction in gastric ulceration. Absorption of GP-λ-D2, if indeed it occurred, therefore appeared to be less than about 2·5 mg/kg (equivalent to about 1 mg total per animal) on the basis of the results in the Table. The minimum dose of GP-λ-D2 showing anti-gastric ulcer activity is 50 mg (Anderson & Soman, to be published); of this, it appears that about 1 mg or less can be absorbed. This suggests that the effect of carrageenan in anti-ulcer experiments is to stimulate some protective mechanism. When 400 mg of C16 was administered intraduodenally in addition to an intravenous injection (groups 4 and 6) the measurable urinary carrageenan did not increase, supporting the conclusion that intraduodenal carrageenan is absorbed in very small amounts.

With the degraded carrageenans it therefore appears that the anti-ulcer effect can be achieved with amounts of carrageenan less than that necessary to give detectable urinary excretion.

Turning to undegraded carrageenan (GP-λ), we have never detected it in urine, although the maximum total dose consumed was 158 mg. GP-λ does have an anti-ulcer effect (Anderson & Soman, to be published), but its high viscosity in solution precludes larger oral or intraduodenal dosage, which the experiments with degraded carrageenan show are necessary to obtain urinary excretion. Thus with the undegraded carrageenans also, doses giving anti-ulcer effect are lower than those which would be required to show urinary excretion. The absorption of undegraded carrageenan, although generally held to be unlikely, is still an open question on the basis of its anti-gastric ulcer effect after intraduodenal administration in the pylorus-ligated guinea-pig (Anderson & Soman, to be published). There are two further points about undegraded carrageenan: first, its molecular size (assuming absence of excessive polydispersity) may well prohibit gastrointestinal absorption; second, intravenous injections of higher doses of undegraded carrageenans in this type of experiment are prevented by extreme toxicity both in rabbits (Anderson & Duncan, 1965) and in guinea-pigs at 1 mg/kg, the animals dying, probably from pulmonary embolism, within half an hour.

That gastrointestinal absorption of degraded carrageenan is possible, helps to explain the more efficient protection (Anderson & Soman, 1965b) against experimental histamine duodenal ulceration the carrageenan gives when administered in drinking water before the histamine rather than after it.

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Short Communication

THE COMPARATIVE PROTECTIVE EFFECTS OF DEGRADED CARRAGEENIN AND ALUMINIUM HYDROXIDE ON EXPERIMENTALLY PRODUCED PEPTIC ULCERATION

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ACUTE peptic ulceration of the duodenum can be produced experimentally in guinea pigs within 24 hours of the administration of a large dose of histamine in beeswax-oil provided a covering dose of an antihistamine is given to protect the animals from acute shock.

Degraded carrageenin, a sulphated polysaccharide (Ebimar) has been shown to react with mucus lining the gastric mucosa and in low concentrations to interfere with peptic digestion. It provides complete protection against duodenal ulceration produced by the above method[1,2]. In the present experiments we have compared its effects on ulcerogenesis with those of aluminium hydroxide.

Methods. Male guinea pigs (300–400 g.) were prepared for experiment by depriving them of food from 5 p.m. on the day before the experiment. The next day at 4.30 p.m. the animals were injected intraperitoneally with promethazine 7·5 mg., followed within half an hour by an intra-muscular injection of histamine acid phosphate 30 mg./kg. in a 10 per cent (w/v) beeswax: arachis oil vehicle. The suspension was made by triturating 1·5 g. histamine acid phosphate (200 mesh) with the vehicle and adjusting the volume to 50 ml.

The animals were studied in groups of five, and the drugs were administered in 2 ml. doses intra-oesophageally at 3 hour intervals from 6 hours before to 21 hours after the histamine injection. Ebimar was given in aqueous solutions containing 20, 10, 5, 1, or 0·2 per cent w/v. Aluminium hydroxide was administered as liquid gel containing the equivalent of 4, 2, or 1 per cent Al_2O_3 w/w. The control group were given 2 ml. of water at the same intervals.

The animals were killed 24 hours after the histamine injection. The stomach and duodenum were removed, fixed in formol saline, and the degree of damage was assessed numerically according to the scheme, Table I.

Results. The degree of damage in the stomach and duodenum in each group of five animals is recorded in Table I. The maximum score if all five animals were severely damaged would be 15 for the stomach, 15 for the duodenum, giving a total score of 30.

It is seen in the control group which received water only that the maximum score was reached in the duodenum. Considerable protection from ulceration in the duodenum was apparent in the groups treated with

10 per cent and 20 per cent sulphated polysaccharide, and in those treated with 2 per cent and 4 per cent aluminium hydroxide.

Less protection was provided with the lower concentrations of each of these drugs. In contrast, however, the sulphated polysaccharide afforded greater protection against stomach ulcers than aluminium hydroxide.

It was observed that considerable caking of aluminium hydroxide had occurred in the stomachs of the groups receiving 4 per cent and 2 per cent gel, but this was not evident in the group receiving 1 per cent gel.

TABLE I

NUMERICAL ASSESSMENT OF THE DEGREE OF GASTRIC AND DUODENAL DAMAGE IN SULPHATED POLYSACCHARIDE AND ALUMINIUM HYDROXIDE-TREATED ANIMALS CONTROL GROUP RECEIVED ONLY WATER

	Sulphated polysaccharide per cent					Aluminium hydroxide gel per cent as Al_2O_3			Water
	20	10	5	1	0·2	4	2	1	
Stomach ..	3–4	2–3	2–3	3	0–1	4–6	8–9	10	9–10
Duodenum	0	3	8	14–15P	12–13P	0	0	6–7	14–15P
Totals ..	3–4	5–6	10–11	17–18P	13–14P	4–6	8–9	16–17	23–24P

Key: 0 No damage,
1 very slight damage, for example, one or two small lesions revealed on careful examination,
2 immediately obvious but not extensive damage,
3 immediately obvious and widespread damage; perforation is indicated by P.

In the sulphated polysacharide treated groups, particularly those receiving the higher concentrations, a fine gelatinous film was observed over the mucosa, especially in the lower third of the stomach and in the duodenum.

Discussion. The method of producing acute peptic ulceration in the above experiments allows the slow release of histamine over a prolonged period. In the guinea pig, histamine stimulates the secretion of a large volume of highly acid gastric juice[3], and it is believed that this factor is primarily responsible for the mucosal damage[4,5]. The results in the aluminium hydroxide series may be said to be in fair agreement with this theory.

From the results of experiments as yet unpublished, we have evidence that in the histamine stimulated guinea pig 2 ml. of 4 per cent aluminium hydroxide gel greatly reduces the acidity of the gastric juice for 2 to 3 hours: 2 per cent gel causes some reduction, but with 1 per cent gel the free acid is only very slightly reduced. It may, therefore, be assumed that over the 27 hour period during which aluminium hydroxide was administered in the present experiments little, if any, free acid is likely to have been present in the stomach or duodenum in the group receiving the 4 per cent gel. This assumption was confirmed by the fact that at post-mortem examination no free acid was detected in the gastric juice in the group receiving 4 per cent gel, but free acid was present in the stomachs of the groups receiving the 2 per cent and 1 per cent gels. As already indicated (Table I), duodenal lesions occurred only in the group given 1 per cent gel. It is noteworthy that despite the accumulation of substantial

amounts of aluminium hydroxide in the stomachs of the group receiving 7 per cent gel, a distinct reaction for free acid (Topfer's reagent) was obtained in the post-mortem juices.

On the basis of the aluminium hydroxide experiments, where increasing damage appears concurrently with increase in free acid, it is not possible to assess the relative importance of the acid and pepsin in the pathogenesis of the ulcerative lesions described. Although these experiments might suggest that the protective action of aluminium hydroxide gel was because of its antacid properties, it must also be appreciated that the anti-peptic activity of this compound may play an important role. Nevertheless, the use of antacids in peptic ulcer therapy is well established and the demonstration of a protective action by the antacid in these experiments perhaps not too surprising. It is interesting to observe on the other hand, that degraded carrageenin which possesses no antacid activity should afford protection to the extent reported.

The evidence we have already obtained about the properties of degraded carrageenin suggests that it has at least two main actions[1,2], and we consider that these actions may be intimately concerned in the protective effects of the sulphated polysaccharide used in these experiments. Degraded carrageenin reacts with mucoprotein in a manner to be expected of polyanionic substances in acid environment. We tentatively conclude that the complex formed between mucoprotein in the mucus lining the stomach and degraded carrageenin serves to enhance the physical protection afforded by mucin. In addition, the degraded carrageenin confers anti-peptic properties on this complex.

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After Dr. Anderson presented the paper there was a DISCUSSION.

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Research Section

A Study of Orally-administered Degraded Carrageenan in the Baboon

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Abstract—No urinary metachromasia was observed when adult baboons were given degraded carrageenan (number-average mol wt 16,000–19,000) in a single dose of 240 mg/kg, which is more than three times the therapeutic dose in man. Even at a dose level of 3 g/kg, more than forty times the therapeutic dose, only 3–6 mg/kg were excreted in the urine over 24 hr, and carrageenan could not be detected in the blood up to 18 hr after dosing, despite the fact that the limit of detection was 5 μg/ml of blood. Urinary metachromasia was determined quantitatively by a spectrophotometric method, and the sulphated polysaccharides responsible for the metachromatic colour were isolated and shown to contain a high proportion of carrageenan, which was characterized by specific rotation, content of ester sulphate and 3,6-anhydrogalactose, infra-red analysis, and paper chromatography of the hydrolysis products. Methods have been developed for the isolation and characterization of degraded carrageenan added *in vitro* to blood, down to a concentration of 100 μg/ml of blood, and for detecting metachromasia down to 5 μg/ml. In the studies undertaken, no detectable amounts of degraded carrageenan were found in the urine of the baboons given three times the therapeutic dose and only very small quantities were detected in specimens of 24-hr urine from animals given the massive dose of 3 g/kg. These results indicate that absorption from the gastro-intestinal tract is unlikely to occur at the levels of degraded carrageenan used therapeutically.

INTRODUCTION

The mechanism of the protective effect of sulphated polysaccharides on experimental ulceration in animals and on peptic ulceration in man is still not definitely understood. Anderson & Soman (1966) showed that oral administration of massive doses (750 mg–4·5 g/kg) of degraded carrageenan to guinea-pigs resulted in detectable urinary metachromasia, suggesting limited gastro-intestinal absorption and excretion of carrageenan.

Eagleton, Watt & Marcus (1968 & 1969) reported that a single subcutaneous injection (400 mg/kg) of degraded carrageenan in guinea-pigs reduced both the volume and the total acid concentration of the gastric juice induced by administration of histamine, and this effect was accompanied by a marked reduction in the incidence and severity of duodenal ulceration. These workers concluded that this inhibition of acid gastric secretion by systemic carrageenan is a major factor in preventing histamine-induced duodenal damage in the guinea-pig.

The object of the present study was to determine whether degraded carrageenan administered orally to the baboon at levels approximating to the human therapeutic level (70 mg/kg) was absorbed from the gut and excreted in the urine.

EXPERIMENTAL

Materials. The degraded carrageenan was prepared from *Eucheuma spinosum* by Laboratoires Glaxo, Paris, (code no. C16/L1927) and gave the following analysis on an anhydrous basis: Total ester sulphate, 37·9% (calculated as SO_3Na); 3,6-anhydrogalactose, 18·1% (as $C_6H_8O_4$); specific rotation ($[\alpha]_D$), + 46·8° (*c* 1·05 g/dl in water); inherent viscosity (η_{inh}), 0·41 dl/g in 0·1 M-sodium chloride solution (*c* 0·21 g/dl, 25°C). The number-average mol wt (M_n) was in the 16,000–19,000 range (W. R. Blakemore & E. T. Dewar, unpublished results), and the weight-average mol wt (M_w, by light-scattering) was 20,000–30,000 (Anderson & Soman, 1966). The material was non-dialysable in Visking dialysis tubing.

Chondroitin sulphate A (chondroitin 4-[potassium sulphate]) and chondroitin sulphate C (chondroitin 6-[potassium sulphate]), prepared by Professor K. S. Dodgson from ox and shark cartilage respectively, were kindly supplied by Dr. D. A. Rees.

Toluidine blue was a George T. Gurr Ltd., London, reagent. A stock solution of this was prepared by dissolving 0·1 g in methanol (70 ml) and diluting to 100 ml with water. A standard solution was prepared by diluting 10 ml of the stock solution to 100 ml with water to give a solution of 0·01% toluidine blue.

Cetyltrimethylammonium bromide (CTAB) was supplied by BDH Chemicals Ltd., Poole, Dorset.

Whole ox blood, obtained from the slaughterhouse in Edinburgh, was citrated (9:1, v/v) with 3·8% (w/v) sodium citrate solution and stored at 0°C.

Trichloroacetic acid "for protein precipitation" was obtained from BDH Chemicals Ltd., Poole, Dorset.

General experimental methods. Infra-red spectra of polysaccharides were measured in potassium chloride discs with Perkin–Elmer 137 and 257 spectrophotometers.

Estimations of ester sulphate and 3,6-anhydrogalactose were carried out as previously described (Black, Blakemore, Colquhoun & Dewar, 1965). The sulphate content of the carrageenans B1 and B2 isolated from urine was determined with 4-chloro-4′-aminodiphenyl reagent (Jones & Letham, 1954), the absorbance being measured at 254 nm in a Uvispek spectrophotometer using 1-cm cells.

Concentration of solutions was carried out in all cases under reduced pressure below 40°C. Samples were dried at 20°C/0·01 mm over phosphoric oxide and all weights are on an anhydrous basis.

Hydrolysis of polysaccharides was carried out with 50% formic acid at 100°C for 18 hr and was followed by paper chromatography using *n*-butanol/ethanol/water (40:11:19, by vol.) as solvent on Whatman no. 1 paper. Sugars were detected with aniline hydrogen phthalate solution (Wilson, 1959) or with silver nitrate–sodium hydroxide reagents (Hough & Jones, 1962).

Animals

Urinary excretion. In the urine experiments, three mature male baboons (*Papio cynocephalus*), weighing 16–18 kg, were starved overnight, sedated with phencyclidine hydrochloride (Sernylan, Parke-Davis, Hounslow, Middx) given in an intramuscular dose of 1 mg/kg, and restrained in a primate chair. A narrow polyethylene sleeve was strapped on to the penis with elastic tape. A piece of sterile polyethylene tubing, adjusted to allow a free flow of urine, was connected to a "Portex" urine-collection bag. The degraded carrageenan, in aqueous solution (100 ml) at 37°C, was given as a single dose by stomach tube. The

animals were restrained in the primate chairs for 24 hr after dosing, during which period they were allowed drinking water *ad lib.* but no food. After the initial sedation, the animals were given further small doses of phencyclidine where necessary to provide continuous light sedation. The 24-hr specimens were analysed immediately, as described below. Control samples of urine were obtained under the same conditions from each animal before treatment with carrageenan. Baboon no. 1 received both the high (3 g/kg) and low (240 mg/kg) dose levels with a 14-day rest period between doses. Baboon no. 2 received only the large dose and no. 3 only the small dose.

Blood level determinations. One male baboon, weighing 6·2 kg, was used for all experiments including the control. The animal was starved overnight and sedated with an intramuscular dose (1 mg/kg) of phencyclidine hydrochloride (Sernylan) and the carrageenan (18·6 g in 40 ml water) was given in a single dose by stomach tube. During the period of the experiments, the animal was allowed drinking water *ad lib.* but no food. A 7-day rest period was allowed between experiments. In one experiment, a 20-ml sample of blood was withdrawn from the femoral vein into a citrated syringe after 3, 6 and 9 hr. In a second experiment, samples were withdrawn at 12, 15 and 18 hr after dosing. The samples were analysed as described below.

Biochemical determinations

Qualitative metachromasia test. A small aliquot of the fresh urine specimen was acidified to pH 4–5 with 2 N-acetic acid, filtered through a sintered glass crucible (porosity 4), and the filtrate (1 ml) was diluted with water (9 ml) and 0·01 % toluidine blue solution (1 ml) was added from a grade 'A' pipette. The absorbance was measured against a water blank at 630 nm in a Uvispek Spectrophotometer using 1 cm cells. A control, in which water (1 ml) replaced the urine, gave an absorbance of 0·71. A test reading of 0·71 ± 0·01 was taken as negative, while an absorbance of less than 0·70 was taken as positive. The test will detect less than 5 μg carrageenan/ml urine.

Quantitative estimation of degraded carrageenan in baboon urine. This colorimetric method is essentially the same as that developed to follow the urinary excretion of sodium laminaran sulphate after injection into experimental animals (Dewar, 1955). A calibration curve was drawn up by treating 10 ml standard solutions, containing 0–100 μg degraded carrageenan, with exactly 1 ml 0·01 % toluidine blue solution and measuring the absorbances at 630 nm in a Uvispek Spectrophotometer using 1-cm cells. The following results were obtained:

Carrageenan (μg):	0	10	20	30	40	50	100
Absorbance:	0·706	0·608	0·502	0·401	0·292	0·190	0·168

The graph of carrageenan concentration against absorbance was a straight line over the range 0–50 μg.

Immediately after the qualitative test for metachromasia was carried out, the urine sample was concentrated to dryness at 35°C in a rotary film evaporator, dried at 20°C/0·01 mm over phosphoric oxide, and the dry urinary solids were stored in the deep-freeze at −20°C and subsequently analysed and processed for the isolation of carrageenan.

For the quantitative estimation of carrageenan, the dry urine sample (*c.* 250 mg) was dissolved in water, the pH was reduced to 5 with a few drops of 2 N-acetic acid, and the solution was made up to 100 ml with water. A 10-ml aliquot of this solution A was treated

with 1 ml 0·01% toluidine blue solution and the absorbance was measured as described above. The carrageenan content was then read off from the calibration curve. Various 10-ml solutions were then prepared by taking aliquots of 1–9 ml solution A and diluting to 10 ml with water, and the absorbances of each diluted solution were measured after the addition of toluidine blue (1 ml). The effect of dilution on carrageenan concentration is shown for the urinary solids (259 mg) from baboon no. 2:

Volume of solution A (%):	10	20	30	40	50	60	70	80	90	100
Carrageenan (μg):	60	65	64	64	58·5	55	49	45	41	38

A graph of volume % against carrageenan concentration was plotted, and the maximum concentration was found to be 65 μg, which was taken as the carrageenan content in 10 ml of solution A. Hence solution A contains 650 μg carrageenan in 100 ml and therefore the carrageenan content of urinary solids equals 0·65 × 100/259 or 0·25%. Because of this dilution effect, which was due to some interference with colour development from urinary constituents, it was necessary to plot the graph of volume of solution A against carrageenan concentration for each estimation and the maximum reading was taken in each case.

Isolation of polysaccharide sulphate B1 from baboon no. 1 dry urinary solids. The dry solids (5·45 g) from baboon no. 1, dosed at the 3 g/kg level, were dissolved in water (150 ml), and the solution was dialysed in Visking tubing against running tap water for 20 hr and then concentrated to dryness at 35°C.

The resultant brown glass (380 mg) was taken up in water (10 ml), the insoluble residue was centrifuged and washed with water (2 × 10 ml), and the clear supernatant and washings were stirred with Amberlite resin IR-120-H (20 ml) for 1 hr to remove all metal ions. After filtration, the resin was washed with water (4 × 30 ml) and the acidic filtrate and washings were neutralized to pH 6 with 0·1 N-lithium hydroxide (11·3 ml). The neutral solution was concentrated to a glass (240 mg). This was redissolved in water (5 ml) and acetone (25 ml) was added. Lithium chloride (240 mg) was added to the colloidal solution to coagulate the precipitate, which was then centrifuged, washed with acetone (2 × 30 ml) and ether (30 ml), and isolated as a light-brown powder (154 mg).

The powder was dissolved in water (10 ml), 20% (w/v) CTAB solution was added dropwise until precipitation was complete, and the polysaccharide sulphate-CTAB complex was centrifuged and washed with water (2 × 10 ml). The complex was dispersed in saturated potassium chloride solution (5 ml), and after 1 hr the insoluble residue was centrifuged and washed with potassium chloride solution (5 ml). The supernatant and washings were treated with ethanol (10 ml), and the copious white precipitate was centrifuged, washed with ethanol (2 × 10 ml) and redissolved in water (40 ml). After dialysis against tap water for 20 hr, the solution was concentrated to dryness, the glass (24 mg) was redissolved in water (5 ml), and acetone (30 ml) was added to give a colloidal solution. The colloid coagulated on the addition of one drop of saturated potassium chloride solution, and the precipitate was centrifuged, washed with acetone (30 ml) and ether (30 ml), and dried at 20°C/0·01 mm over phosphoric oxide to give carrageenan B1 as a cream-coloured powder (21·1 mg, 0·39% of dry urine).

In the same way, dry solids (3·05 g) from baboon no. 2 after dosing at the 3 g/kg level yielded carrageenan B2 (6·6 mg, 0·22%).

These carrageenans were analysed for ester sulphate and 3,6-anhydrogalactose content and characterized by their specific rotation and infra-red spectra and by paper chromatography of their hydrolysis products.

Isolation and characterization of carrageenan from ox blood (in vitro). Degraded carrageenan (50·2 mg) in water (5 ml) was added to citrated ox blood (55·5 ml, containing 50 ml blood), followed by supersaturated potassium chloride solution (500 ml), with stirring, and the mixture was stirred overnight. Trichloroacetic acid (10%, w/v) in saturated potassium chloride solution (150 ml) was added slowly and the mixture was stirred for a further 4 hr. The precipitate of blood proteins was centrifuged and washed with 3% (w/v) trichloroacetic acid in saturated potassium chloride solution (2 × 250 ml), and the clear supernatant and washings were neutralized to pH 8 with solid sodium hydrogen carbonate. After 3 days' dialysis against running tap-water, the solution was concentrated to dryness. The yellow solid (1·48 g) was redissolved in water (40 ml) and centrifuged, and 20% (w/v) CTAB was added to the supernatant until precipitation was complete. The complex was centrifuged, washed with water (2 × 40 ml), dissolved in saturated potassium chloride solution (10 ml) and reprecipitated with ethanol (10 ml). The polysaccharide sulphate–potassium chloride precipitate was centrifuged, washed with ethanol (2 × 40 ml), dissolved in water (40 ml) and dialysed overnight against running tap-water to remove potassium chloride. The dialysed solution A was concentrated to 5 ml, acetone (30 ml) was added, and the colloid was coagulated with one drop of saturated potassium chloride solution. The final precipitate, containing the carrageenan, was centrifuged, washed with acetone (40 ml) and ether (40 ml), and dried at 20°C/0·01 mm over phosphoric oxide to a powder (37·8 mg; 75·3%, based on weight of carrageenan). Found: 3,6-anhydrogalactose, 15·1%; ν_{max} (cm^{-1}), *inter alia*, 940–930 (3,6-anhydrogalactose), 850–840 (axial sulphate), 810–800 (3,6-anhydrogalactose 2-sulphate).

In a control experiment, 50 ml blood gave no precipitate with CTAB in the absence of degraded carrageenan. A similar control of 50 ml baboon blood also gave no precipitate.

Isolation and estimation of carrageenan added to blood in low concentrations. When the concentration of added carrageenan in the blood was reduced below 10 mg/100 ml, the quantity of carrageenan recovered by the above procedure was too small (<3 mg) for identification by chemical and infra-red analysis. Consequently, the final dialysed solution A containing the carrageenan (see above) was concentrated to dryness, the residue was redissolved in a suitable volume of 0·1 N-hydrochloric acid and the sulphated polysaccharide content was estimated with toluidine blue using the method of MacIntosh (1941).

The *in vitro* determinations of the baboon blood levels were carried out similarly except that 20 ml volumes of blood were used instead of 50 ml.

RESULTS AND DISCUSSION

Urinary excretion

The qualitative test of the 24-hr urine specimen from two baboons given 3 g degraded carrageenan/kg showed the presence of sulphated polysaccharides in both specimens. Urinary metachromasia was then determined quantitatively and the results are shown in Table 1, which also includes details of the two experiments carried out with the much lower dose level (240 mg/kg).

Thus at the 3 g/kg level, the urinary excretion of carrageenan amounted to 0·19% of the total oral dose (50·6 g) in baboon no. 1 and 0·1% in baboon no. 2, i.e. between 3 and 6 mg/kg body weight. This is an exceedingly small amount, when one considers that this dose level is more than forty times the human therapeutic dose (*c.* 70 mg/kg). When the degraded carrageenan was given at a dose level of 240 mg/kg, about 3–4 times the therapeutic dose, no

Table 1. *Study of urinary excretion of carrageenan in the baboon*

Baboon no.	Baboon weight (kg)	Total carrageenan dose (g)	Dose level (g/kg)	Total volume of urine (ml)	Qualitative metachromasia reaction	Urinary solids (g)	Carrageenan in dry solids (%)	Concn in urine (μg/ml)
1	16·5	50·6	3·1	570	Positive	21·8	0·43	168
2	15·7	50·6	3·2	875	Positive	20·9	0·25	60
1	15·8	3·8	0·24	410	Negative			
3	18·3	4·2	0·23	1075	Negative			

Table 2. *Analyses of carrageenans B1 and B2*

Polysaccharide sulphate	Yield (% of dry urine)	Carrageenan content (%)	$[\alpha]_D$ (*c.* 0·5 in H_2O)	SO_3Na (%)	3,6-Anhydrogalactose (%)	Important bands in infra-red spectrum (cm^{-1})	Sugars on hydrolysis
B1	0·39	88	+ 34±4°	26·4	9·6	940–930 850–840 810–800	Gal, GA R_{gal} 1·3 R_{gal} 1·5
B2	0·22	87	—	33·0	10·8	940–930 850–840 810–800	Gal, GA R_{gal} 1·3 R_{gal} 1·5

Gal = galactose; GA = glucuronic acid; R_{gal} = distance travelled on chromatogram relative to galactose.

urinary metachromasia was detected, although the toluidine blue test will detect less than 5 μg carrageenan/ml urine.

The polysaccharide sulphate responsible for the metachromatic colour was isolated from both samples of urine at the 3 g/kg dose level and found to contain a high proportion of carrageenan (Table 2). The yields of both sulphated polysaccharides were in good agreement with those determined colorimetrically (Table 1). The specific rotation of B1 was less than that (+47°) of the original carrageenan but was still clearly positive, in marked contrast to the negative rotations of chondroitin sulphates A, B and C (Brimacombe & Webber, 1964). The quantity of B2 isolated was too small for the measurement of optical rotation. The ester sulphate content of B1 was less than that (37·9%) of the original carrageenan, indicating some contamination, although the sulphate content of B2 was appreciably higher; chondroitin sulphates A, B and C (as their dipotassium salts) have a theoretical sulphate (as SO_3Na) of only 19·3%.

The 3,6-anhydrogalactose contents of B1 and B2 indicate that the polysaccharide sulphates contain 53–60% of carrageenan. The presence of 3,6-anhydrogalactose in B1 and B2 is definite evidence of carrageenan, for it was found that colour formation with chondroitin sulphate A (and presumably other mucopolysaccharides) was negligible in this test.

Further evidence of carrageenan was obtained from the infra-red spectra. The spectra of B1 and B2 (Table 2) were characteristic of carrageenan from *E. spinosum* (Black *et al.* 1965) with peaks at 940–930, 850–840, and 810–800 cm^{-1}, the last being due to 3,6-anhydrogalactose 2-sulphate (Anderson, Dolan, Penman, Rees, Mueller, Stancioff & Stanley, 1968). Chondroitin sulphate A showed bands at 940–930 and 860–850 cm^{-1} (axial sulphate), but gave no absorption at 810–800 cm^{-1}. Chondroitin sulphate C absorbs at 820 (equatorial sulphate) but not at 810–800 cm^{-1}.

Hydrolysis of the two polysaccharides with 50% formic acid and subsequent paper chromatography showed the main monosaccharide component to be galactose, which is absent from hyaluronic acid, chondroitin sulphate and heparin. Several other components, however, were also present, notably a sugar with R_{gal} 1·5 and another with R_{gal} 1·3, which is probably 2-acetamido-2-deoxy-D-galactose derived from chondroitin sulphates (probable contaminants of both B1 and B2). Glucuronic acid was a minor component.

Blood levels

If it is assumed that all the carrageenan excreted in the urine over 24 hr, i.e. 3–6 mg/kg body weight, was contained in the blood volume (*c.* 60 ml/kg body weight) of the baboon at one time, then the maximum concentration of carrageenan in the blood would be 5–10 mg/100 ml. Obviously, this concentration is probably an upper limit but it formed a useful basis for our *in vitro* experiments with both ox and baboon blood.

The first task in the present study was to determine whether carrageenan at this concentration could be isolated from blood and characterized, and all the initial *in vitro* experiments were carried out with citrated whole ox blood. The decomposition of any blood protein carrageenate complexes by addition of supersaturated potassium chloride solution to the blood containing the added carrageenan can be represented by:

$$\underset{\text{Complex}}{ROSO_3^-/R^1NH_3^+} + \underset{\text{Carrageenan}}{KCl} \longrightarrow ROSO_3K + \underset{\text{Protein}}{R^1NH_3Cl}$$

Addition of trichloroacetic acid completed the precipitation of proteins, after which exhaustive dialysis was required to free the solution from salts. After precipitation of the

Table 3. *Analyses of carrageenans isolated from ox blood*

Concentration of carrageenan in blood (mg/100 ml)	Yield (% of added carrageenan)	3,6-Anhydro-galactose (%)	Important bands in infra-red spectrum (cm^{-1})		
100	75	15·1	940–930	850–840	810–800
20	69	13·4	940–930	850–840	810–800
10	66	—	940–930	850–840	810–800

polysaccharide sulphate with CTAB, the CTAB complex was decomposed with potassium chloride, and the polysaccharide sulphate was precipitated with acetone and analysed.

In a control experiment, comparable blood without added carrageenan gave no precipitate with CTAB. The results for three concentrations of carrageenan in the blood are shown in Table 3.

The combination of bands in the infra-red recorded in Table 3 is characteristic of this type of carrageenan, which was confirmed at the first two concentrations by the presence of 3,6-anhydrogalactose. The infra-red analysis showed the presence of some contamination, particularly in the material isolated at the 10 mg/100 ml level.

The results of the spectrophotometric estimation of polysaccharide sulphate obtained from carrageenan added to blood in concentrations below 10 mg/100 ml are given in Table 4, for

Table 4. *Spectrophotometric estimation of carrageenan isolated from blood*

Type of blood	Concentration of carrageenan in blood (mg/100 ml)	Recovery of carrageenan (%)
Ox	10	54
	5	54
	2	51
Baboon	2	47
	0·5	*c.*38

three concentrations of carrageenan in ox blood and two concentrations in baboon blood. The lowest level of detection by this procedure, using toluidine blue, is 5 μg/ml blood. This concentration gives a very slight turbidity on the addition of CTAB, while no turbidity is obtained at lower concentrations.

A male baboon was given a single oral dose of 3 g degraded carrageenan/kg body weight, and 20 ml blood was withdrawn from the femoral vein at 3-hr intervals and treated as described for the *in vitro* experiments. In the first experiment, no turbidity was detected after 3, 6 or 9 hr while in the second experiment, there was no evidence of turbidity in the blood samples withdrawn after 12, 15 or 18 hr. The concentration of carrageenan in the blood thus did not reach 5 μg/ml.

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Étude du carragène dégradé administré par voie orale au babouin

Résumé—Aucune métachromasie de l'urine n'a été observée chez des babouins adultes après l'administration d'une dose unique de 240 mg/kg de carragène dégradé (moyenne numérique du poids moléculaire: 16.000–19.000). Cette dose est plus que le triple de la dose thérapeutique employée pour l'homme. Même au niveau de 3 g/kg, soit plus de quarante fois la dose thérapeutique, seulement 3 à 6 mg/kg étaient excrétés par l'urine en 24 h et on ne pouvait déceler le carragène dans le sang au cours des 18 h suivant l'administration, ceci nonobstant le fait que la limite de détection était de 5 μg/ml de sang. La métachromasie de l'urine a été déterminée quantitativement par une méthode spectrophotométrique. Les polysaccharides sulfatés responsables de la couleur métachromatique ont été isolés et on y a trouvé une forte proportion de carragène, qui a été identifié par son pouvoir rotatoire spécifique et sa teneur en sulfate estérifié et en 3,6-anhydrogalactose, par analyse infrarouge et par la chromatographie sur papier des produits d'hydrolyse. Des méthodes ont été élaborées pour isoler et identifier jusqu'à un taux de 100 μg par ml de sang le carragène dégradé ajouté *in vitro* au sang et pour déceler la métachromasie jusqu'à 5 μg/ml. Aucune quantité décelable de carragène n'a été découverte, au cours des recherches décrites ici, dans l'urine des babouins qui avaient reçu trois fois la dose thérapeutique et l'on n'en a décelé que de très faibles quantités dans des échantillons d'urine excrétée pendant 24 h après l'administration par des animaux qui avaient reçu la dose massive de 3 g/kg. Ces résultats indiquent qu'aux doses utilisées en thérapeutique, le carragène n'est probablement pas absorbé par le tractus gastro-intestinal.

CARRAGEENAN AND THE HUMAN GUT

SIR,—Marcus and Watt[1] reported last year that carrageenan (a substance obtained from seaweeds) caused ulceration of the intestinal tract in certain animals, and suggested that it might be a hazard in man. This prompts me to report my own experience with degraded carrageenan ("Ebimar").

My knowledge of the product dates back to 1958, when I was requested by the French Ministry of Health to conduct the toxicological, pharmacological, and clinical investigations required before approval by the French authorities. As a consequence of this investigation, I treated 200 peptic-ulcer patients with 5 g. of ebimar daily. Throughout the period of their treatment, which ranged from 6 months to 2 years, I followed up these patients personally. Radiological examinations were done at intervals of not more than 4 months.

Ulcerative colitis is so well defined a clinical entity that it is unlikely that any competent clinician would overlook its presence in his patients. The association of peptic ulcer and ulcerative colitis in the same patient is extremely rare; and my work on peptic ulcer has made me particularly interested in patients in whom these two disease-entities might coexist. Yet, in none of the 200 cases of peptic ulcer treated with ebimar was there any evidence of additional gastrointestinal disease. Many of these patients are still under my surveillance; and I and my colleagues have since prescribed ebimar to hundreds of ulcer patients without noting any colonic symptoms.

A further experiment designed to determine whether degraded carrageenan is absorbed from the colon in patients with ulcerative colitis is relevant. 10 such patients were given 10 g. ebimar daily for 10 days—equivalent to twice the therapeutic dose. No absorption could be demonstrated

1. Marcus, R., Watt, J. *Lancet*, 1969, ii, 489.

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2 : 414

at this dosage, and regular sigmoidoscopic examinations revealed no adverse changes with respect to colonic ulceration or mucosal bleeding. I believe that if, as has been suggested, carrageenan has a deleterious effect on the colon, this would have become obvious in the above investigations.

My 12 years' experience of the clinical use of degraded carrageenan,[2,3] as well as the toxicological and pharmacological investigations I have carried out, lead me to the conclusion that in human beings it is free from side-effects and from risk of toxicity to the colon.

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THE INTERACTION BETWEEN CARRAGEENAN AND THE FIRST COMPONENT OF COMPLEMENT

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Davies (1) recently reported that carrageenan, a sulfated polysaccharide, is a potent *in vitro* and *in vivo* inhibitor of complement (C′) activity. By the use of the intermediate products formed by the interaction of specific sites (S) on sheep erythrocytes (E), rabbit antibody to boiled sheep erythrocyte stromas (A) and guinea pig complement (C′), he showed that EAC′1, EAC′1,4, EAC′1,4,2 and the C′3 complex are not inhibited by carrageenan. With the aid of R reagents (2) he concluded that a component acting in the hemolytic system prior to C′1 may be affected by carrageenan. He also reported that one-half hour following intravenous injection of carrageenan, 5 mg/kg, blood C′ of guinea pigs fell to less than 1% of the original level. This report deals with an investigation of the mechanism of inhibition of C′ *in vivo* and *in vitro* by carrageenan. Our results indicate that carrageenan interacts with and precipitates C′1 *in vitro*, that C′1 can be recovered from these precipitates, and that carrageenan prevents interaction between EA and C′1. In our hands, however, carrageenan was not as potent an inhibitor of C′ *in vivo* as reported (1).

MATERIALS AND METHODS

Source and preparation of E, C′, purified guinea pig (g.p.) C′2, EAC′1a,4 and of veronal buffered saline (VBS) have been described (2). Low ionic strength VBS was prepared according to Rapp and Borsos (3). C′1a was separated from whole g.p. serum and its activity was measured by its reactivity with EAC′4 as described by Borsos & Rapp (4).

Esterase activity of C′1a using *p*-tosyl-L-arginine methyl ester (TAMe, Nutritional Biochemical Corporation) as substrate was determined with the aid of an automatic pH titrator (Radiometer, Copenhagen). The measurements were performed at 37°C using 5.0 ml of 0.5 M TAMe in VBS (lacking gelatin and divalent cations) to which 1.0 ml of C′1a was added. The acid was titrated with 0.02 N NaOH to pH 7.4. This procedure is a modification of the method described by Ratnoff and Lepow (5).

SeaKem type 21 carrageenan, obtained from Marine Colloids, Inc., New York, was dissolved in boiling 0.15 M NaCl, then cooled to room temperature. Sodium azide, which has no measurable effect on C′ action, was added as a preservative in a final concentration of 0.005 M. Animals were injected with freshly prepared carrageenan solutions lacking sodium azide.

RESULTS

Formation of cellular intermediates with C′ inhibited by carrageenan. Davies' conclusion that carrageenan affects a complement component acting prior to C′1 was based on the observation that EA which remain unlysed in the presence of C′ inhibited by carrageenan were lysed by R1 in the absence of carrageenan. It is known, however, that R1 contains a small amount of C′1, and thus is capable of lysing cells which contain SAC′4. If one assumes that a small fraction of C′1 is capable of interacting with EA in the presence of carrageenan, a small proportion of the cells may acquire SAC′1a,4. Because of the transferability of C′1, however, a much larger proportion of cells will acquire SAC′4. Thus, after separating these cells from carrageenan, they would be susceptible to lysis by R1. There is no need, therefore, to postulate that a C′ component exists which acts before C′1. The assumption that a small amount of C′1 reacts with EA in the presence of carrageenan was tested by examining such EA for the presence of SAC′1a,4 and SAC′4. One-milliliter portions of VBS, each containing 3 C′H50, were mixed with 1-ml portions of VBS, each containing a different amount of carrageenan, and the mixtures were incubated for 10 min at 30°C. At this time, 4.5 ml of VBS were added to each mixture followed immediately

...ly by 1 ml of EA containing 5 × 10⁸ cells/ml. These mixtures were then incubated at 37°C for 1 hr and centrifuged, and the optical densities of the supernatant fluids were determined at a wave length of 541 mμ in the Beckman DU spectrophotometer. The unlysed cells were washed and resuspended to a concentration of 5 × 10⁸ cells/ml. To determine the nature of sites that are left on these cells, the following experiments were performed.

Test for SAC'4. One half-milliliter aliquots from these unlysed cell suspensions were exposed to 0.5 ml of purified C'1a (supplying 4000 effective molecules of C'1a per cell) for 10 min at 30°C. At that time 0.5 ml of C'2 was added, supplying approximately 2000 C'2 molecules per cell. After 10 min at 30°C, 5 ml of C'EDTA (g.p. C' diluted 1/50 in VBS containing 0.01 M Ethylenediaminetetraacetic acid (EDTA)) were added and the cells were incubated for 90 min at 37°C, at which time the extent of lysis was determined in the usual manner.

Test for S.1C'1a,4. One half-milliliter aliquots from each unlysed cell suspension were exposed to purified C'2 and to C'3 as described above, except that no C'1a was added.

The results presented in Table I indicate that a proportion of EA surviving treatment with C' inhibited by carrageenan carry SAC'1a,4 and SAC'4 sites. The number of these sites depends on the degree of inhibition. For example, 3C'H50 inhibited by 160 μg of carrageenan lysed 4.2% of the original EA, and 19.7% of the surviving cells carried SAC'4 on their surface. Some of these surviving cells carry SAC'1a,4 as well, for 5% were lysable by C'2 and C'3. On the other hand, 3C'H50 inhibited by 100 μg of carrageenan lysed 47.5% of the original EA, 79.2% of the surviving cells carried SAC'4 on their surface, and 11.2% of the surviving cells were lysable by C'2 and C'3. These observations justify the assumption that in the presence of inhibitory levels of carrageenan C'1 may react with EA to form intermediate products.

Precipitation of C'1 from whole serum by carrageenan. When a solution of carrageenan is added to whole serum a precipitate is formed and the C'1 activity of the serum is reduced. The possibilities were considered that the precipitate may contain C'1 and that active C'1 could be recovered from the precipitate. Accordingly the following experiment was performed.

Four milligrams of carrageenan in 1.5 ml of solution were allowed to react with 5.0 ml of g.p. serum for 5 min at room temperature. The resulting cloudy suspension was centrifuged for 30 min at 0°C at 28,000 × G. The supernatant liquid was then poured off and the precipitate was recentrifuged for 10 min at 28,000 × G. The resulting small amount of supernatant liquid was drained and the tube was wiped free of all traces of liquid. The precipitate was dissolved in 1.0 ml of 3.5 M NaCl at room temperature and, after adding 0.2 ml of H_2O, the solution was centrifuged at 900 × G for 10 min at room temperature. Next, 1.0 ml of 3.0 M $(NH_4)_2SO_4$ was added to the supernatant fluid followed by incubation for about 5 min at 37°C. The resulting precipitate was recovered by centrifugation and was redissolved in 1.0 ml of 0.15 M NaCl. About 40% of the initial C'1 activity was recovered from the precipitate (Table II).

Interaction of carrageenan with purified guinea pig C'1a. Equal volumes of a dilution of C'1a were mixed with equal volumes of VBS containing a measured amount of carrageenan. The mixtures were incubated for 10 to 15 min at 30°C and the residual C'1a activity was determined. Figure 1 depicts an experiment in which constant amounts of C'1a were mixed with varying amounts of carrageenan, whereas Figure 2 shows the results of an experiment in which constant amounts of carrageenan were reacted with varying amounts of C'1a.

TABLE I

Formation of cellular intermediates by C' inhibited by carrageenan

C'H50	3	3	3	3	3	3
Carrageenan (μg)	160	140	120	100	80	0
Lysis of EA (%)	4.2	6.7	19.3	47.5	80.9	100
Lysis of surviving cells						
by C'1a, C'2, C'3 (%)	19.7	28.8	54.9	79.2	100	
by C'2 C'3 (%)	5.0	5.7	11.1	11.2	17.7	

TABLE II

Precipitation and inhibition of C′1 by carrageenan and recovery of C′1 from the precipitate

	Total C′1 units
Guinea pig C′	175,000
C′-carrageenan[a]	30,000
C′1 recovered from precipitate	67,000

[a] Refers to C′1 activity measurable in uncentrifuged guinea pig serum-carrageenan mixture.

It can be seen from Figures 1 and 2 that carrageenan is an effective inhibitor of purified C′1a.

The effect of carrageenan on esterase activity of C′1a preparations. The results in Table III indicate that 4 mg of carrageenan, which inhibited 67% of the hemolytic activity of a preparation of purified C′1a, was incapable of inhibiting the hydrolysis of TAMe by C′1a. Thus carrageenan appears to interact with C′1 at a site distinct from the enzymatically active site of the molecule. This observation is in accord with Davies' finding that neither EAC′1 nor EAC′1,4 is inhibited by carrageenan. It was then postulated that carrageenan blocks that site of C′1 which interacts with SA. This was tested in the next experiment.

Interaction of carrageenan with EAC′1a,4. C′1 dissociates from antibody-antigen complexes. This dissociation can be virtually prevented by lowering the ionic strength from the usual 0.15 to $\mu = 0.065$. The effect of carrageenan on SAC′1a,4 activity in VBS of $\mu = 0.15$ and of $\mu = 0.065$ was examined. The experiment was performed with one effective molecule of C′1 per EAC′1a,4 and an excess of C′4. Two 6-ml portions of EAC′1a,4 containing 7.5×10^7 cells/ml were centrifuged. One pellet was resuspended in 6 ml of VBS, $\mu = 0.15$ and the other in 6 ml of VBS, $\mu = 0.065$, both at 30°C. At zero time 0.06 ml of a solution containing 1 mg of carrageenan/ml was added to each cell suspension. One half-milliliter samples removed at convenient time intervals were centrifuged, resuspended in VBS, $\mu = 0.065$, and C′2 and C′3 were added (4). Results are shown in Figure 3. The curves

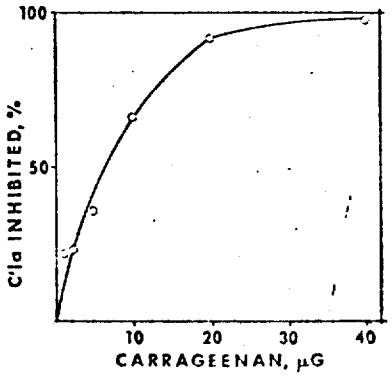

Figure 1. The effect of varying amounts of carrageenan on 1.5×10^{10} effective molecules of C′1a.

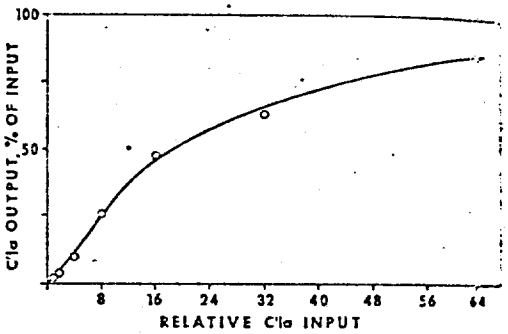

Figure 2. The effect of 12.5 μg of carrageenan on varying amounts of C′1a. One relative unit of C′1a = 3.0×10^3 effective molecules.

TABLE III

Effect of carrageenan on esterase activity of C′1a

	Without Carrageenan	With Carrageenan
Esterase activity (μmoles acid/hr)	6.0	6.0
Hemolytic activity (effective molecules/ml)	3.0×10^{12}	1.0×10^{12}

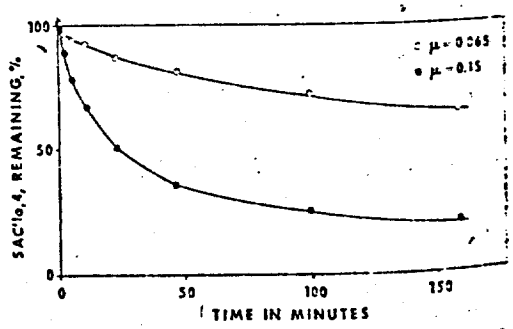

Figure 3. The effect of carrageenan on activity of EAC′1a,4 at two ionic strengths

were normalized to an initial activity of 100%. From the initial rates, it was calculated that SAC'1a,4 activity at $\mu = 0.15$ disappears six times more rapidly than at $\mu = 0.065$. This showed that the rate of inactivation of C'1a activity depended on the extent (or rate) of dissociation of C'1a from SAC'4. This is interpreted to mean that the site or sites of C'1a which interact with carrageenan are not available when C'1a is in association with SAC'4.

Effect of carrageenan on in vivo C' of guinea pig. Guinea pigs were injected intravenously with carrageenan, 8 mg/kg. Prior to injection, a small sample of blood was removed by heart puncture from each animal. Control animals received 2 ml/kg of 0.15 M NaCl, a volume equal to that volume of carrageenan solution given to experimental animals. Thirty minutes after the injection of carrageenan another small volume of blood was withdrawn by heart puncture. As an additional control in certain experiments the second blood sample was obtained under light ether anesthesia.

In a typical experiment the blood samples were allowed to clot for 15 min at room temperature and the clot and blood cells were removed by centrifugation in the cold at 800 × G for 10 min. The sera were kept at 0°C. Determinations of C'1 and C'4 titers were performed as soon as possible, but not more than a few hours after the blood was obtained.

In Table IV the results of three experiments are presented. It is seen that the loss in whole C' titer is usually not more than 50% compared with the preinjection titers. Since our results are in contrast with those of Davies, we sought some explanation for this discrepancy. One possibility was that the EA itself may serve as a source of C'1. However, identical results were obtained when C' activity was determined with EA treated with EDTA. Such treatment removes C'1 which may have been associated with EA. In addition, C'1 transfer experiments (EA to EAC'4) detected no C'1 activity with our preparations of EA. In one experiment (2a, Table IV) in which aliquots of the clotted blood were intentionally kept for a number of hours at room temperature before measuring C' activity, C' levels about 80% lower than control titers were obtained in carrageenan-treated animals. Therefore, prolonged storage of blood at room temperature before titration was avoided. As expected, the major

TABLE IV

Effect of carrageenan on C' in vivo[a]

Exp. No.	Guinea Pig No.[b]	Treatment	C'H50		C'1		C'4	
			Pre	Post	Pre	Post	Pre	Post
1	1	S	361	334	34,000	30,000	102,000	75,000
	2	C	300	170	34,000	3,800	83,000	57,000
	3	C + E	341	211	27,000	3,800	70,300	25,000
	4	S + E	254	248	31,000	33,000	42,000	48,000
2	5	S	294	200	27,000	27,000	41,000	46,000
	6	C	326	139	25,000	2,500	50,000	23,000
	7	C + E	312	166	54,000	5,000	52,000	35,000
	8	S + E	254	224	14,000	8,000	45,000	37,000
2a[c]	5	S	294	294	27,000	18,000	41,000	58,000
	6	C	326	60.5	20,000	300	50,000	36,000
	7	C + E	312	83.3	54,000	600	52,000	46,000
	8	S + E	254	273	14,000	6,000	45,000	48,000
3	9	C	240	120	30,000	300	29,000	18,000
	10	C + E	244	167	30,000	2,000	32,000	16,000
	11	S + E	222	154	16,000	12,000	17,000	11,000

[a] S, injected with 0.15 M NaCl; C, injected with carrageenan; E, second blood sample was obtained under light ether anesthesia; pre, blood obtained before treatment; post, blood obtained 30 min after treatment.

[b] Guinea pigs nos. 1 to 8 weighed 1000 g, guinea pigs nos. 9 to 11, 250-300 g.

[c] see text for explanation

effect of carrageenan was on C′1 with a loss ranging from 90 to 99% of initial C′ activity. It has been postulated on the basis of indirect evidence that whole C′ titers are relatively insensitive to large changes in C′1 concentration (6). Our experiments provide direct evidence in support of this postulate. There was little or no loss of C′4 activity following injection of carrageenan. Light ether anesthesia had little effect on either C′, C′1 or C′4 activities (Table IV).

DISCUSSION

Studies on the biologic action of C′ would be facilitated if an efficient, nontoxic inhibitor of C′ were available. Carrageenan seemed to fulfill these requirements. We have found that, though carrageenan interacts with and inhibits C′1, its capacity to reduce C′ levels *in vivo* is not great enough to be of value for studies on the biologic action of C′. There are several factors which limit the usefulness of carrageenan. The components of C′ which are known to react stoichiometrically are C′1, C′4 and C′2. Of these, C′1 is generally present in the highest concentration in all mammals tested thus far (7). We have calculated that it is practically impossible to administer sufficient carrageenan *in vivo* to bind enough C′1 so that C′ activity is virtually eliminated. Part of this difficulty is due to the fact that a relatively large reduction in C′1 content causes a relatively small change in C′ titer (see Table IV, Experiment 2a). In addition, Davies (1) has pointed out that carrageenan causes a prolongation of bleeding time and we have found this effect becomes more evident with increasing concentrations of carrageenan.

We can offer no explanation for our inability to achieve the degree of inhibition of C′ *in vivo* that Davies obtained beyond the possibility that different strains of guinea pigs have different concentrations of C′1. Nevertheless, carrageenan is a valuable tool for studies on the structure of C′1, for we have obtained evidence that carrageenan interferes with that part of C′1 which appears to be responsible for its binding to sensitized erythrocytes (EA). In addition, our observations that carrageenan precipitates C′1 from serum and that active C′1 can be obtained from such precipitates indicates that carrageenan may be valuable in the purification and immunochemical analysis of C′1.

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SUMMARY

Carrageenan inhibits the hemolytic activity of C′1a by interfering with the capacity of C′1 (and of C′1a) to become bound to sensitized erythrocytes. Carrageenan has no effect on the esterase activity of C′1a. The degree of inhibition of C′ activity *in vivo* is too small to be of value in studies on the biologic action of C′.

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Spectrum and Possible Mechanism of Carrageenan Cytotoxicity

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Carrageenan, a sulfated polygalactose which suppresses established delayed hypersensitivity *in vivo*, is shown to be cytotoxic to macrophages but not to lymphocytes *in vitro*. This cytotoxicity depends on the carrageenan concentration and degree of lysosomal differentiation but is independent of serum. Survival of macrophages in the presence of carrageenan can be enhanced temporarily by corticosteroids. Ultrastructural studies reveal that carrageenan is readily taken up by macrophages and stored in lysosomes, which subsequently swell and rupture, apparently resulting in cell death. The presence of corticosteroids temporarily retards lysosome swelling. It is suggested that carrageenan may exert its cytotoxic effect by causing osmotic rupture of lysosomes. The possible immunologic significance of these findings is discussed. (Amer J Path 64:387–404, 1971)

CARRAGEENAN is a long-chain, high-molecular-weight, sulfated polygalactose obtained from marine plants. Recently, this polysaccharide has been shown to suppress delayed hypersensitivity in tuberculin-sensitive guinea pigs.[1] In addition, carrageenan inhibits the complement system,[2,3] activates Hageman factor (factor XII)[4] and at high concentration acts as an anticoagulant.[4,5] The mechanism by which carrageenan suppresses delayed hypersensitivity has yet to be elucidated.

There is an intimate cooperation between lymphocytes and macrophages in the elicitation of the delayed hypersensitivity response.[6–12] Both cell types in the response are thought to be of bone marrow origin,[13,14] and appear to interact sequentially. Cells of the lymphoid series "recognize" and interact with specific antigen and are stimulated to produce soluble "mediators" such as migration inhibitory factor (MIF)[8,10,12,14] and a chemotactic factor[15] which may directly affect macrophage mobility and function. The accumulation of macrophages at sites of delayed hypersensitivity reactions is thought to play a substantial role in the tissue damage associated with these reactions.[8–12]

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and immediately placed in plastic petri dishes (Falcon Plastics) containing 10–15 ml of TC 199 with heparin (10 units/ml) and 20% NBCS. The splenic capsules were incised with a razor blade and the cells gently shaken loose. Care in this procedure was found to be essential for maximal initial survival of lymphocytes. The cells were centrifuged in the cold at 800 rpm for 9 minutes and washed 3 times with warm TC 199 (without heparin) containing 20% NBCS. Ten million cells in 1 ml were plated on capped plastic 25-sq-cm T flasks. The flasks were gassed with 5% CO_2–95% air, capped tightly and placed on a rocking platform at 37 C for 2 hours. The cells adhering firmly after this time had the morphology of macrophages and accounted for 6–10% of the population. The supernate containing nonadherent cells was considered a lymphocyte-rich population and consisted of approximately 98% lymphocytes, as determined by morphologic criteria.

Determination of Viability

1. Exclusion of the dyes erythrosin B [17] or trypan blue [18] by cells in suspension or adherent to the surface of plastic T flasks was used as a criterion of viability. Routinely, 400 cells were observed in a hemocytometer or *in situ* by exploiting the optical properties of the plastic T flasks. All results were expressed as percent survival.

2. It was apparent that viability determinations by dye exclusion for suspension of macrophages might be biased by selective adherence of some subpopulation to the sides of the plastic culture tubes. To exclude this possibility, we utilized the following modification of a method of Stewart and Ingram.[19] At time zero, test suspensions of macrophages (both control and experimental) were divided into enough separate 1-ml aliquots (each containing 1×10^6 cells) for duplicate determinations at each time period. To each tube at the appropriate time, 0.5 ml of pronase (5 mg/ml) (Calbiochem, Los Angeles, Calif) was added and the mixture incubated for 20 minutes at 37 C. Pronase digests *entire* nonviable cells but only the cytoplasm of viable cells while sparing the nuclei. Thus, the number of nuclei after pronase digestion corresponds to the number of viable cells. Clearly this procedure frees any adherent cells from the side of the tube. To facilitate counting of nuclei, cytologic debris was lysed by the addition of 1 ml of Cetrimide (5 mg/ml) (cetyltrimethylammonium bromide, Eastman Organic Chemicals, Rochester, NY) in which the nuclei are stable. The nuclei were stained with Turk's solution and counted in a hemocytometer. Thus, the number of nuclei (*ie*, number of viable cells) at any time period divided by the number of nuclei (*ie*, number of viable cells) at zero time is recorded as the percent survival at that time period (we are assuming initial survival to be 100%; this is a realistic assumption since viability in parallel controls determined by dye exclusion was never less than 97%).

Ultrastructural Studies

Aliquots of cells were taken from suspensions of cells (both guinea PEC and mouse spleen lymphocytes) in which no serum was present (to avoid sampling errors due to selective adherence). The cells were fixed in a glutaraldehyde–osmium tetroxide mixture, then postfixed in uranyl acetate, as described by Hirsch *et al.*[20] They were then dehydrated in a graded series of alcohols, embedded in Araldite, stained with lead citrate and examined in an AEI EM-6B electron microscope. Suspensions of carrageenan alone in saline (10 mg/ml) were fixed, processed and studied in the same way as the cell suspensions.

Results

Lymphocyte Survival

In three separate experiments, there was no difference in survival between control cultures of spleen lymphocytes and those incubated with concentrations of carrageenan cytotoxic for macrophages (Table 1). Only those experiments in which the controls maintained a high rate of survival during the entire time period are included in the table. Clearly, using the other experiments would make the task of assessing cytotoxic effects of carrageenan versus adverse culture conditions impossible. However, even in the experiments with poor initial viability, survival was no different in controls than in those incubated with carrageenan. Survival was determined in all cases by dye exclusion.

Macrophage Survival

Serum Factors. The presence of serum in the culture medium was not necessary for the cytotoxic effects of carrageenan on macrophages in suspensions. Table 2 lists cell survival in paired experiments containing either TC 199 with 20% NBCS v/v or TC 199 alone with varying concentrations of carrageenan. Both dye exclusion (erythrosin B) and the pronase-cetrimide digestion technic (see Materials and Methods) gave similar results, indicating that selective adherence did not bias the results. Similar toxicity, independent of serum factors, was observed in macrophages adherent to the surface of plastic T flasks.

Influence of Carrageenan Concentrations. The results of these experiments are summarized in Table 2 and the Text-fig 1. At a concentration of carrageenan of 0.6 mg/ml, a maximal rate of cytotoxicity

Table 1—Lymphocyte Survival with Varying Concentrations of Carrageenan*

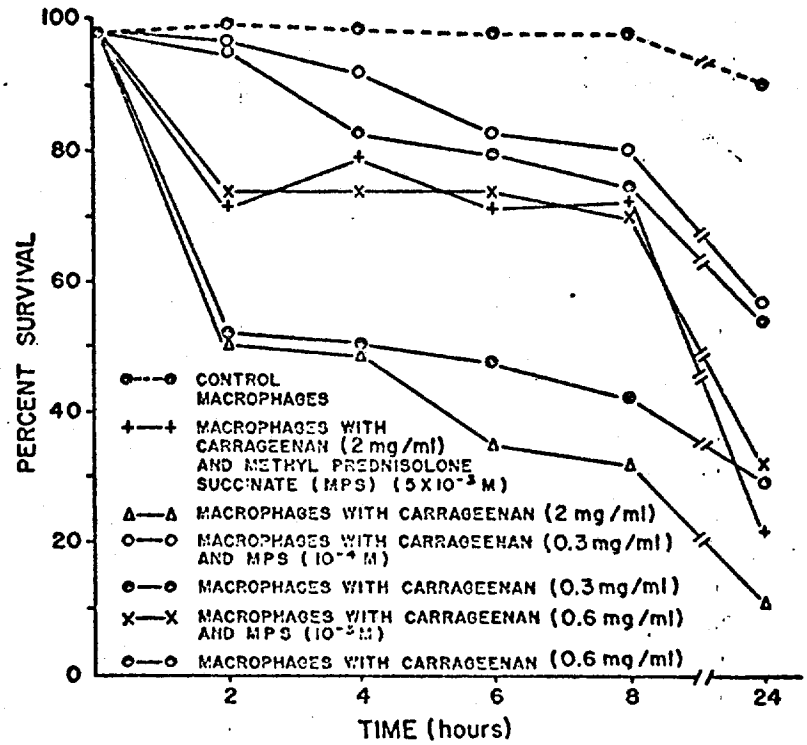
Time (hours)	Lymphocyte survival (% with SD)			
		Carrageenan		
	Control	0.1 mg/ml	0.6 mg/ml	2 mg/ml
0	98.3 ± 1.06	98.3 ± 1.06	98.3 ± 1.06	98.3 ± 1.06
2	96.6 ± 1.70	96.3 ± 1.11	96.0 ± 1.82	95.7 ± 1.69
4	97.0 ± 2.08	94.8 ± 0.97	95.0 ± 1.68	95.5 ± 2.20
6	95.3 ± 0.27	94.3 ± 0.47	94.3 ± 0.85	95.4 ± 2.15
8	93.3 ± 0.29	93.3 ± 1.08	94.3 ± 1.39	94.0 ± 2.14
10	93.5 ± 0.13	92.0 ± 1.62	93.0 ± 0.81	92.5 ± 0.13

* All measurements done on 3 separate spleens at 3 separate times except for 10-hour results which represent 2 experiments.

Table 2—Effect of Concentration of Serum and Carrageenan on Macrophage Survival

Time and technic	Macrophage survival (mean % and range)*							
			Carrageenan					
	Control		0.3 mg/ml		0.6 mg/ml		2 mg/ml	
	Serum†	No serum‡	Serum	No serum	Serum	No serum	Serum	No serum
0 hours								
PC§	100 ± 0.0¶				100.0 ± 0.00		100.0 ± 0.0	
EB‖	97.7 ± 1.16	97.5 ± 1.50	99.0 ± 0.0	99.9 ± 00.0	98.0 ± 0.0		99.5 ± 0.5	96.2 ± 1.2
2 hours								
PC	98.4 ± 1.12				59.8 ± 1.4		55.6 ± 0.6	
EB	97.3 ± 2.50	96.5 ± 2.51	94.5 ± 0.5	95.0 ± 0.0	50.5 ± 0.5		52.0 ± 1.0	51.2 ± 0.9
5 hours								
PC	94.8 ± 1.42				54.5 ± 0.5		42.1 ± 1.1	
EB	96.0 ± 2.00	96.0 ± 1.25	82.5 ± 0.5	83.5 ± 0.5	50.0 ± 0.5		49.9 ± 0.5	50.6 ± 0.3
7 hours								
PC	92.6 ± 1.40				43.4 ± 1.5		35.3 ± 0.5	
EB	95.3 ± 0.79	96.7 ± 0.9	80.0 ± 0.00	84.1 ± 1.0	47.0 ± 0.5		34.0 ± 1.0	32.1 ± 0.0
24 hours**								
PC	90.0 ± 2.0				18.6 ± 0.8		21.7 ± 1.2	
EB	90.0 ± 0.0		53.5 ± 2.5		23.2 ± 1.2		16.0 ± 2.0	

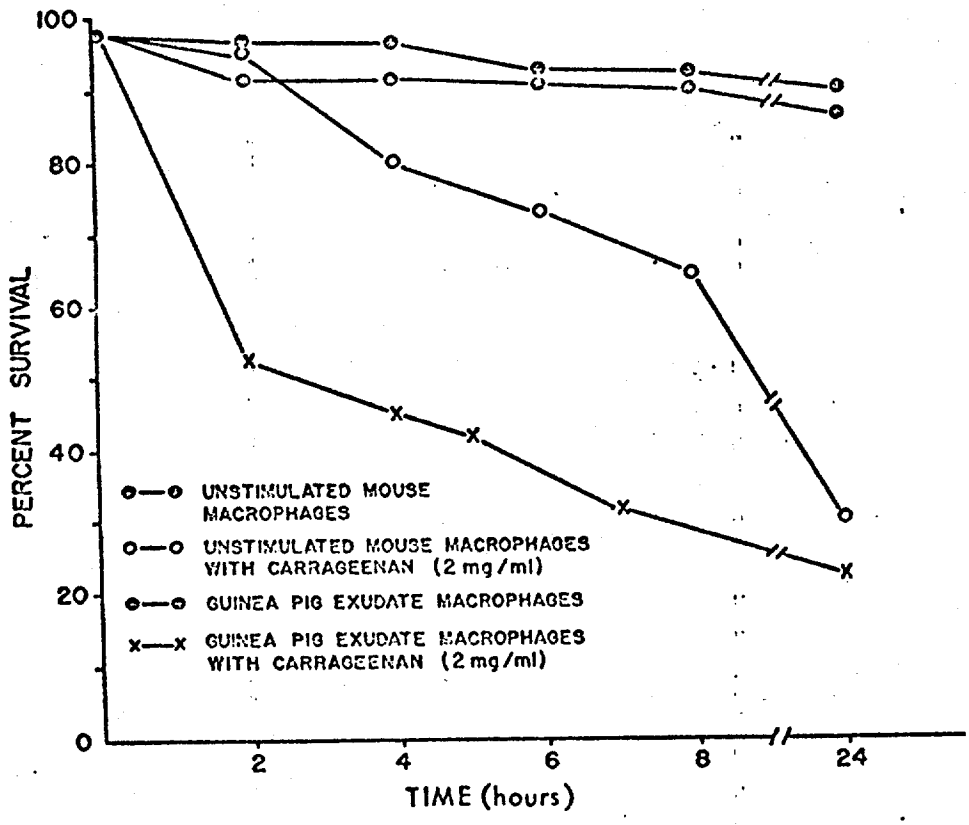
* Each column represents two separate experiments, two determinations per experiment (see Materials and Methods section).
† Serum = TC 199 with 20% NBCS v/v.
‡ No Serum = TC 199 alone.
§ PC = survival measured by pronase digestion technic (see materials and methods section).
‖ EB = survival measured by exclusion of erythrosin B.
¶ Initial survival is assumed to be 100% in this method.
** Since macrophages do not survive well without a protein source, no determinations were done on the "no serum" group after 7 hours.



TEXT-FIG 1—Comparison of the effects of varying concentrations of carrageenan and methylprednisolone alone and together on survival of guinea pig peritoneal exudate cells. Survival is expressed as percent of total cells. Curve marked control represents survival of control cells with or without MPS.

was achieved. Concentrations at or above this level effected a rate of cytotoxicity twofold greater than concentrations below this level. No increase in toxicity was noted at concentrations above 0.6 mg/ml. Similar results were obtained in suspensions or in cultures of macrophages adherent to the surfaces of plastic T flasks. As cited above, the presence or absence of serum was not a factor in toxicity.

Survival of Macrophages from Unstimulated Mice Compared with Those from Guinea Pig Exudate. Text-fig 2 shows a typical experiment comparing guinea pig exudate peritoneal macrophages with unstimulated mouse peritoneal macrophages, both incubated in presence of 2 mg/ml carrageenan. Since approximately 40% of normal mouse peritoneal cells are lymphocytes by morphologic criteria, a macrophage-rich preparation was prepared by incubating the cells in plastic T flasks and discarding the nonadherent cells. The results depicted thus compare survival of guinea pig exudate macrophages in suspension



TEXT-FIG 2—Comparison of survival of unstimulated mouse peritoneal macrophages with guinea pig peritoneal exudate cells. Survival is expressed as percent of total cells.

with that of unstimulated mouse macrophages adhering to plastic T flasks. While survival at 24 hours is approximately the same for both species, the unstimulated macrophages fare better at earlier times.

Protective Effect of Methylprednisolone. Text-fig 1 depicts the protection afforded to suspensions of macrophages incubated with carrageenan and methylprednisolone succinate. Clearly protection, *ie,* survival, increases proportionally with the concentration of methylprednisolone. Parallel experiments were performed with macrophages adhering to plastic T flasks. At concentrations of methylprednisolone greater than 5×10^{-3} M, the macrophages became detached from the plastic surface and assumed a uniformly spherical conformation. This phenomenon of detachment and spherical conformation is consistent with other reports.[21]

Ultrastructural Studies

Ultrastructural studies of suspensions of carrageenan in saline in the absence of cells revealed an amorphous material with a fibrillar component (Fig 2A). In each experimental situation where carrageenan was present, an identical amorphous material with a fibrillar component was seen both extracellularly (Fig 2B) and intracellularly in membrane-lined vacuoles (Fig 3 and 4) and partially membrane-lined cytoplasmic "lakes" (Fig 5 and 6). Similar material was not seen in control preparations or in experiments in which cells were heated at 56 C for 30 minutes. In addition, cells were noted in the process of engulfing this material (Fig 2B). For the above reasons, this amorphous material with a fibrillar component was presumed to be carrageenan (Fig 2A).

Aliquots of macrophage suspensions cultured in the presence of 0.6 mg/ml of carrageenan with or without 5×10^{-4} M methylprednisolone succinate (MPS) were studied after 2 and 5 hours incubation. Control MPS-treated macrophages seemed to have more vesicular cytoplasmic profiles but in all other respects they were similar to the non-MPS controls (Fig 1). Phagocytosis of carrageenan by macrophages was noted in each experimental situation (Fig 2C). Because the electron microscope is not a quantitative tool, it was impossible to compare the amount of uptake of carrageenan in the two groups. However, uptake of carrageenan was observed in approximately the same percentage of MPS-treated cells as in cells not treated with MPS. After uptake of carrageenan, the two groups exhibited similar cellular adaptations and differed only in the proportion of cells involved.

The carrageenan was stored in vacuoles presumed to be secondary lysosomes (Fig 3 and 4). The intravacuolar appearance presumably reflected the action of degradative enzymes and varied from fibrillar (essentially unchanged from extracellular carrageenan) to amorphous, an end-stage form. Many vacuoles contained material that was intermediate between the fibrillar and granular. It was not unusual to notice in the same cell several vacuoles containing carrageenan in different stages of degradation. The size of the carrageenan-containing vacuoles varied from small to markedly swollen. Cells with this appearance exhibited no nuclear or mitochondrial abnormalities. It appears also that products of carrageenan degradation accumulate in nonmembrane-bound or partially membrane-bound cytoplasmic lakes (Fig 5 and 6).

Exudate and spleen lymphocytes in all experimental situations and at each time period appeared structurally unaltered. After 5 hours'

incubation with concentrations of 0.6 mg/ml of carrageenan, lymphocytes do not take up carrageenan in amounts detectable by electron microscopy. Some fibrillar material was seen adhering to the surface of some lymphocytes. However, it was impossible to determine whether this was an artifact of fixation or actual interaction of carrageenan with lymphocyte membranes.

Discussion

The fact that carrageenan is toxic *in vitro* to macrophages and not to lymphoid cells, which generally are nonphagocytic,[22] suggests that uptake of this polysaccharide is necessary for the realization of its toxic effect. While finding large amounts of carrageenan in macrophages, we found none in splenic or exudate lymphocytes, which is consistent with toxicity in the former but not the latter. Further, macrophages rapidly sequester endocytosed polysaccharides,[23] and notably carrageenan,[16] into lysosomes. In addition, the toxic effects of carrageenan may be partially or totally prevented for some time by methylprednisolone, a synthetic glucocorticoid known to stabilize lysosomal membranes.[24] Further, there is greater survival at early times in the unstimulated peritoneal macrophage of the mouse, which has an immature lysosomal complex.[25,26] Cultured in the presence of NBCS, this lysosomal complex rapidly matures [25,26] and it is thus of interest that survival at 24 hours in the mouse macrophage system is comparable to that in guinea pig PEC. All the above strongly suggest that the lysosome is the site of initial damage by carrageenan and consequently it is those cells with a well-developed lysosomal complex which are selected for the cytotoxic action of carrageenan.

There are two possible mechanisms for lysosomal damage, neither of which is mutually exclusive. First is a toxic effect of carrageenan on lysosomal membranes. Because carrageenan has inhibitory effects on the complement system [2,3] and activates Hageman factor,[4] we investigated the possibility that carrageenan interacted with some serum factor to produce a lysosomal toxin. Since the results show toxicity to be independent of the presence of serum, we must conclude that any lysosomal membrane toxicity, if present, is not serum-mediated. However, these studies do not rule out the formation of a toxic complex consisting of carrageenan and some protein adsorbed to the plasma membrane or present in the lysosome. Further, it is clear that the exploration of the relation of carrageenan and Hageman factor would best be done in a plasma rather than serum-supplemented medium. Since toxicity was present in the absence of both serum and plasma,

these experiments were not considered central to the present consideration.

A second possibility involves osmotic rupture of lysosomal membranes with consequent release of digestive enzymes and cell death. Cohn and Ehrenreich [23] have demonstrated lysosomal swelling due to ingestion of oligosaccharides, which cannot be broken down quickly by lysosomal glycosidases into molecules below a molecular weight of 360. Membranes are impermeable to molecules above this size and retention of such molecules in lysosomes in this undergraded form can exert an osmotic effect.

Carrageenan belongs to a class of polysaccharides known as *gel-forming polysaccharides*. Its primary structure is an alternating copolymer $(A - B)_n$ where $B = \beta$-D-galactose 4-sulfate and $A =$ 3,6-anhydro-α-D-galactose or its 2-sulfate.[27] X-ray diffraction studies have suggested a double helical conformation with covalent interchain bonds. Thus carrageenan, unlike most long-chain biologic polysaccharides, has both a secondary and tertiary structure.[27]

It is possible that this complex molecular structure may be particularly resistant to rapid digestion by lysosomal glycosidases. The micrographs in Fig 3 and 4 show a similarity in structure between extracellular (Fig 2A–C), and intracellular carrageenan, indicating that much of this material has not been digested down to the level where it is freely diffusible and thus not osmotically active. Thus, we propose the following sequence:

Carrageenan is taken up by endocytosis and transported to secondary lysosomes in the same way as other polysaccharides.[23] Because of its unique secondary and tertiary structure, it is resistant to total digestion to osmotically inactive molecules. Lysosomal swelling ensues and results in rupture of the lysosomal membrane with consequent release of carrageenan and lysosomal hydrolases into the cell sap. It is this lysosomal rupture which results in the partially membrane-lined lakes of carrageenan in the cytoplasm. The fact that steroid-treated macrophages have generally smaller carrageenan-containing lysosomes may be the result of decreased uptake. However, the phenomenon of normal endocytosis in the presence of steroids has been reported by others.[21] Perhaps the interaction of steroids with the lysosomal membrane [21] gives mechanical stability which retards osmotic swelling and results in smaller vacuoles. Eventually the protection of steroids is lost. This may be the result of cellular metabolism of the steroid. Alternatively, the steroid may be unable to "stabilize" lysosomal membranes in view of the fact that the cell persists in incorporating more

carrageenan, which upon incomplete degradation produces additional increases in osmotic swelling. Clearly a combination of the above may be possible.

It is of interest that carrageenan is not inhibitory to thymidine incorporation by sensitized lymphocytes exposed to the specific antigen *in vitro*.[28] Thus, it is doubtful that carrageenan exerts its depression of established delayed hypersensitivity by inhibiting antigen stimulation of lymphocytes.

If lymphocytes took up carrageenan by endocytosis and stored it in lysosomes, it is conceivable in this situation that carrageenan would exert a cytotoxic effect similar to that which it has in macrophages. In this regard, the presence of lysosomes in lymphocytes has been reported by Diengdoh and Turk.[29] It has also been noted that lymphocytes stimulated by phytohemagglutinin (PHA) to undergo blast transformation are phagocytic.[30] It also is probable that "inactive" small lymphocytes must undergo blast transformation and division before engaging in immune reactions.[30] All of these facts strongly imply that while carrageenan may be nontoxic to nonphagocytic, resting lymphocytes *in vitro*, it may be toxic to the lymphocytes that have become phagocytic in the process of blast transformation. Thus, *in vivo*, it is conceivable that carrageenan may suppress delayed hypersensitivity by being toxic to macrophages and antigen-stimulated lymphocytes.

A still unanswered problem presently under investigation in our laboratory is the site of action of carrageenan *in vivo*. Since the bone marrow is the source of both cell types in the delayed response,[13,14] this may be a likely site.

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THE CARCINOGENIC ACTION OF CARRAGEENIN IN RATS

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ROBERTSON and Schwartz (1953) showed that "carrageenin", (a sulphated polygalactose made from the sea weed, Irish moss, *Chondrus crispus*,) when injected subcutaneously into guinea-pigs would induce the proliferation of fibroblasts and the formation of a connective tissue granuloma. This technique was used to study the biochemistry of collagen formation by Jackson (1956*a* 1956*b*, 1957), Slack (1956, 1957, 1958), Robertson and Hinds (1956), Robertson, Hiwett and Herman (1959). The histology of the lesions produced has been described by Williams (1957) and Benitz and Hall (1959).

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METHODS

Female Wistar rats 60 days old and between 100 to 130 g. in weight were injected into the left flank with 5 ml. of 1 per cent w/v carrageenin in 0·9 per cent salt solution. Three days later they were injected into the right flank with 1·5 mg of caproylethyleneimine in 0·15 ml. arachis oil, and on the 7th day a similar quantity of caproylethyleneimine was injected into the middle of the carrageenin induced mass in the left flank. Some preliminary experiments had confirmed that 7 days after the injection of carrageenin there were numerous young fibroblasts present in the mass of new fibrous tissue which had been induced. The two doses of ethyleneimine were given on separate days to avoid loss of rats from the toxicity of this reactive compound. In all four batches of rats were used:

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In the preliminary experiment (group A) 16 rats were killed 307 days after the injection of carrageenin. At autopsy it was noted that the mammary tissue on the left side differed markedly in appearance from that on the right side. The right side showed a little, rather thin mammary tissue embedded in normal subcutaneous fat, typical of a rather senile, resting mammary gland. On the left side the mammary tissue was much thicker, there was dilatation of the small vessels, the fat was yellow or brown and was sometimes creamy in consistence and the tissue was adherent to the skin. Because of these changes the remaining animals were allowed to survive until they had produced tumours or were ill or died. No rats produced sarcomas on the right side, but 9 out of 18 rats surviving more than 400 days produced sarcomas on the left side. The main experiment was therefore set up consisting of groups B, C and D (Table I). In this experiment all the sarcomas arose in the left flank, but it showed the unexpected result that carrageenin alone would induce sarcomata. We were not able to prove by the detailed statistical analysis, given below, that a single dose of an acylethyleneimine given into the carrageenin-induced granuloma had a significant synergistic effect in producing more sarcomas or shortening the induction period.

Statistical analysis

Table I shows the tumour incidence and the mean induction time of tumours of the 4 groups of animals surviving more than 400 days. Since experiment A was terminated after 650 days, the mean induction times of tumours for groups B, C and D up to 650 days were also computed to allow comparison with experiment A. The table shows that there are no statistically significant differences between the three groups B, C and D after 650 days. The principal difference

TABLE I.—*Tumour Incidence and Mean Induction Times*

	Preliminary experiment Group A	Main experiment Group B	Main experiment Group C	Main experiment Group D	Groups A and B pooled
Treatment					
To right flank	Caproylethylenimine	Caproylethylenimine	Acetylethylenimine	Saline	Caproylethylenimine
To left flank	Carrageenin + caproylethylenimine	Carrageenin + caproylethylenimine	Carrageenin + acetylethylenimine	Carrageenin + saline	Carrageenin + caproylethylenimine
Number of rats with sarcomas on right side	0	0	0	0	0
Number of rats surviving more than 400 days	18	23	26	39	41
Number of rats developing sarcomas on left side in 650 days	9	3	3	6	12
Mean induction time truncated at 650 days and standard error	590·6 ± 20·8	635·5 ± 10·5	637·4 ± 6·4	640·6 ± 4·7	615·2 ± 11·5
Number developing sarcomas on left left side in 825 days	—	5	9	11	14
Mean induction time truncated at 825 days	—	784·3 ± 22·1	749·4 ± 20·9	766·0 ± 17·1	724·3 ± 23·1

TABLE II.—*Expected Numbers Developing Tumours*

	Group A	Group B	Group C	Group D	Group A and B pooled
Number of rats surviving more than 400 days	18	23	26	39	41
Number of sarcomas developing in 650 days	9	3	3	6	12
Expected number per 1000 developing sarcomas in 650 days and standard error	653·8 ± 140·8	150·4 ± 81·0	135·6 ± 73·9	199·3 ± 73·4	350·2 ± 83·9
Expected number in experiment developing sarcomas in 650 days	11·8	3·5	3·5	7·8	14·4
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Expected number in experiment developing sarcomas in 825 days	—	14·6	14·0	21·3	29·6

Note: The expected numbers above refer to the expected numbers per thousand that would develop sarcomas if there were no deaths from other causes.

in mean induction times at 650 days is between group A and the other three groups. The difference between groups A and B is not significant at the 5 per cent level. The difference between groups A and C, and A and D exceed twice their standard errors, but it is not possible to assess the precise significance of their difference owing to uncertainty as to the degrees of freedom that should be attached to the standard errors. Sukhatme's d test (Fisher and Yates, 1953 Table V. 1) indicates, however, that the differences are near the 5 per cent level. Further the larger standard deviation of group A (which is approximately given by $\sqrt{9 \times 20{\cdot}8} = 62{\cdot}4$) compared with the other groups suggests that the form of the distribution of induction times for group A may be different from that of groups B, C and D.

After 825 days, there is no statistically significant difference between the mean induction times for groups B, C and D. If the results from groups A and B are pooled there is no statisticant difference between the mean induction time for this group and the remaining two groups C and D.

Table II shows, assuming no deaths from other causes, the number of rats per thousand that would be expected to have developed tumours after both 650 and 825 days. After 650 days there is no statistically significant difference between groups B, C and D, but there is between these groups and group A, the latter developing more tumours per thousand. After 825 days there is again no statistically significant difference between groups B, C and D.

The figures were computed according to Irwin (1949)

Macroscopic changes induced by carrageenin

A mass was palpable in the region of the injection site for about two weeks. Loss of hair was noticed over the injection site commencing about three weeks after the injection and persisting in many cases for many months. When tumours began the palpable mass usually increased very rapidly in size. In most cases it was necessary to kill the animal within three weeks of first detecting the tumour but sometimes a small mass was felt for some weeks and then suddenly increased rapidly in size. The changes in the mammary tissue at autopsy included a visible increase in the amount of mammary tissue on the left side, a fluid or creamy consistency of the fat, numerous small vessels in the injection area and adherence of the mass to the skin.

Histological appearances of the changes induced by carrageenin

The essential changes induced by carrigeenin can be described under 6 headings.

1. *The early changes.*—These consist of an accumulation of polymorphonuclear leukocytes, macrophages with phagocytosed polysaccharide and fibroblasts. Fig. 1 shows the appearance one week after the injection and shows many macrophages and numerous young fibroblasts. Later mature collagen fibres are in greater evidence. The early changes have been described in detail by Williams (1957) and Benitz and Hall (1959) and need not be further elaborated here.

2. *Degeneration of mammary gland epithelium* was seen in progress even more than two years after the carrageenin injection. Fig. 2 shows the typical appearance of the unaffected mammary tissue on the right side. Note a small cluster of gland alveoli separated by much adipose tissue from its nearest neighbour. Note also the thin walled normal capillaries. Fig. 3 shows that the changes

induced by carrageenin are still progressing 813 days after injection. Note in the centre of the field mammary epithelial cells in process of degeneration. In other parts of the field the empty spaces represent gland alveoli which have degenerated. There is a great increase of the intra-lobular fibrous tissue.

3. *Invasion of degenerated gland alveoli by fibroblasts.*—Fig. 4 shows a good example of this in the lower part of the field; note how the space left by a mammary gland alveolus which has degenerated has now been filled with a group of fibroblasts. A somewhat earlier stage of this process is seen near the middle of the right margin of this picture.

4. *Thickening of the walls of the capillaries and arterioles.*—This change which can be seen in Fig. 3 and 4 is illustrated more clearly in Fig. 5, 6 and 7. Fig. 5 is stained with methyl violet and shows the thick layer of hyaline material surrounding the endothelial lining of the capillary. There are many mast cells present immediately outside this hyaline material. With methyl violet the hyaline material is faintly metachromatic and the mast cells stain deep blue. Fig. 6 is from the same block and is stained with P.A.S. At the bottom of the picture the thickened capillaries and the mast cells are very clear. In the top right hand corner, there are capillaries with walls as much as 4 times as thick as the diameter of the capillary lumen. Fig. 7, stained with haematoxylin and eosin, shows a small arteriole with a greatly thickened wall running in a tortuous spiral diagonally across the field. Other thickened vessels can be easily identified in other parts of this picture.

5. *Involvement of small nerves in the fibrous tissue.*—The fibrous tissue replacement of the mammary tissue appears to enmesh many small nerves. Fig. 8 shows a typical field. Fig. 9 shows four of the changes induced by carrageenin: —the cells lining the mammary gland alveoli have become pale staining, with vacuolated cytoplasm and small pyknotic nuclei; there is definite replacement of gland cells by fibroblasts in the central alveolus; the capillary walls are thickened and a small nerve has been caught in the area of fibrosis.

6. *Sarcomatous changes.*—Zones of precancerous proliferation and early sarcomatous changes were seen in some of the sections. Fig. 10 is from a zone 1 to 2 mm. across which contains many large cells with heavy staining and abnormal nuclear formations. Fig. 11 shows part of an early fibroblastic sarcoma. Most of the large tumours were of this type but some were fibro-sarcomata containing collagen fibres stainable by Van Gieson and some were more anaplastic in type. One typical myxosarcoma was seen.

One rat with a large fibroblastic type sarcoma had bilateral deposits at the lower poles of both kidneys, see Fig. 12 which shows the tumour cells infiltrating between the kidney tubules. A lymphosarcoma of the thymus was also noted in another rat.

DISCUSSION

The original purpose of the experiment was to show whether a single dose of an acylethyleneimine, a simple but short acting carcinogen, would produce a tumour if placed in contact with young, actively growing fibroblasts. The whole basis of the experiment was invalidated by the quite unexpected finding that carrageenin by itself would produce sarcomata. Careful statistical analysis of the results did not show a significant synergistic activity of the single dose of ethyleneimine and the carrageenin.

The long-lasting changes produced by the carrageenin were also quite unexpected. Williams (1957) and Benitz and Hall (1959) described the histological changes produced by carrageenin in the first 28 days following injection. The author has been unable to find any description in the literature of the late effects of injected carrageenin. The degenerative changes in mammary gland epithelium and replacement by fibroblasts are what might have been anticipated but the curious changes in the small vessels were a surprise. The small vessels appear like pipes with thick walls, the hyaline material stains slightly pink with methyl violet, red with periodic-Schiff, eosinophil with haematoxylin and eosin and blue with the picro Mallory stains. Frequently there are many mast cells surrounding these vessels. It is possible that the chronic changes in the mammary gland are due to the interference with its blood supply, but macroscopically the carrageenin-injected tissue appears to be much more vascular than the control gland.

The loss of hair over the injection site is also something of a mystery. It might be due to irritation and scratching by the animal—irritation due to alterations of blood supply or the inclusion of nerve fibres in the mass of fibrous tissue. On the the other hand the hair loss might be due to the interference with the subepidermal layer of elastic tissue and hair follicles. Williams (1957) notes that intradermal injection of carrageenin in the guinea-pig produced after 5 days a degeneration of the collagenous fibres in the dermis, and despite partial remodelling in the later stages the dermis suffered a substantial loss of its collagenous tissue. My clinical impression is that some of the changes in the skin

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EXPLANATION OF PLATES

FIG. 1.—Subcutaneous tissue of a rat 7 days after injection of carrageenin only; note the numerous macrophages and fibroblasts. H. & E. ×570.

FIG. 2.—Control mammary tissue (on right side) 722 days after start of experiment. Note thin walled capillary and much interlobular adipose tissue. Picro Mallory. ×475.

FIG. 3.—Degeneration of mammary gland epithelium with increase of intralobular fibrosis and hyaline thickening of capillary walls seen 454 days after injection of carrageenin + caproylethyleneimine. Picro Mallory. ×360.

FIG. 4.—Degenerated mammary gland epithelium being replaced by fibroblasts, see gland alveolus at bottom centre, also hyaline thickening of capillary walls, 545 days after injection of carrageenin + acetylethyleneimine. Picro Mallory. ×360.

FIG. 5.—Hyaline thickening of capillary walls which are surrounded by mast cells, same rat as Fig. 3, 454 days after injection of carrageenin + caproylethyleneimine. Methyl violet. ×360.

FIG. 6.—From the same rat as Fig. 3 and 5, stained with P.A.S. ×360.

FIG. 7.—Tortuous arterioles and capillaries with hyaline thickening of their walls, 803 days after injection of carrageenin, only. H. & E. ×360.

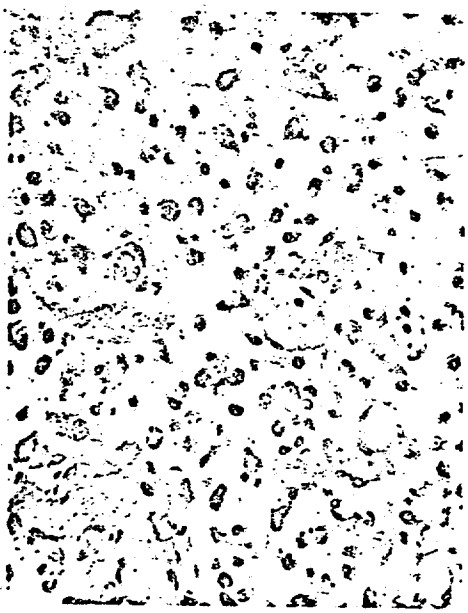
FIG. 8.—A number of fine nerves caught in the fibrous tissue replacing the mammary tissue 813 days after injection of carrageenin only. Picro Mallory. ×360.

FIG. 9.—Typical carrageenin changes—pale vaculated mammary gland epithelium with pyknotic nuclei with replacement by fibroblasts, upper centre; there is a small nerve caught in the process and some hyaline thickening of capillary walls 562 days after injection of carrageenin + acetylethyleneimine. Picro Mallory. ×360.

FIG. 10.—Section from a small zone 1-2 mm. in diameter in which sarcomatous transformation has taken place, 545 days after injection of carrageenin + acetylethyleneimine. H. & E. ×360.

FIG. 11.—Typical fibroblastic type of sarcoma 618 days after carrageenin injection + caproylethyleneimine. The tumour 40 × 15 × 15 mm. in size appeared 28 days previously. H. & E. ×570.

FIG. 12.—Tumour invading right kidney. There is a pleomorphic-cell sarcoma in left flank 722 days after injection of carrageenin + acetylethyleneimine. H. & E. ×360.



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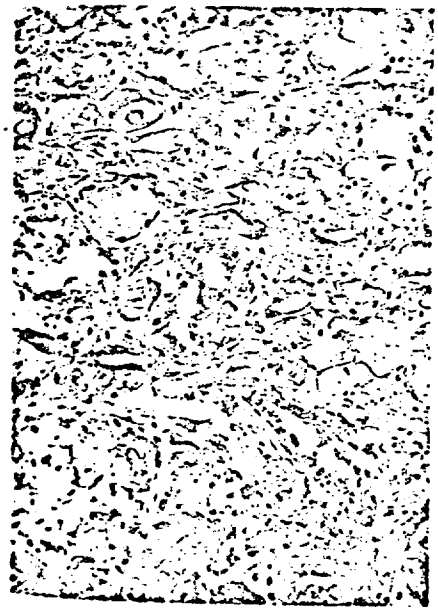

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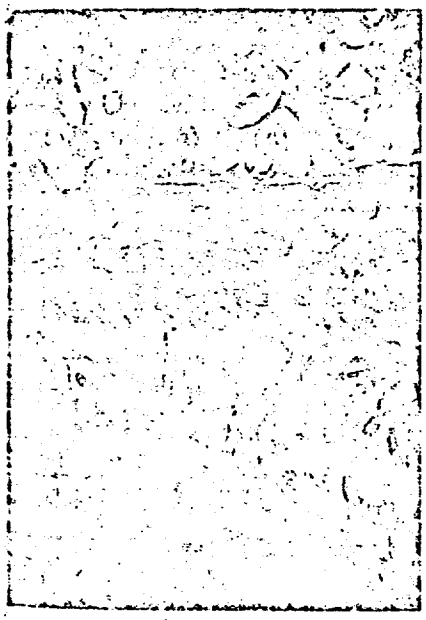

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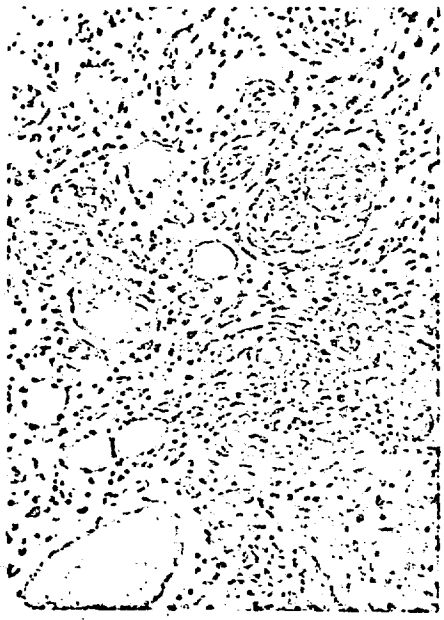

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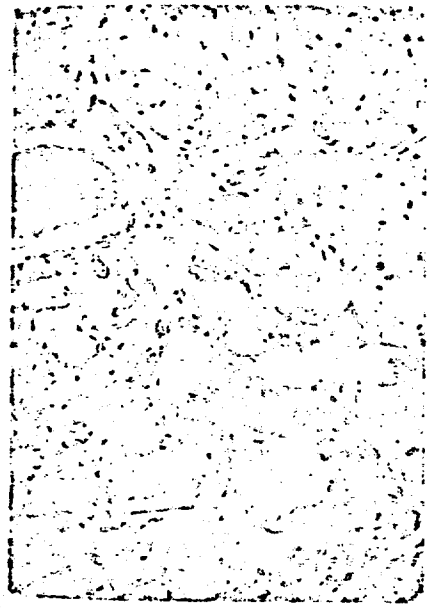

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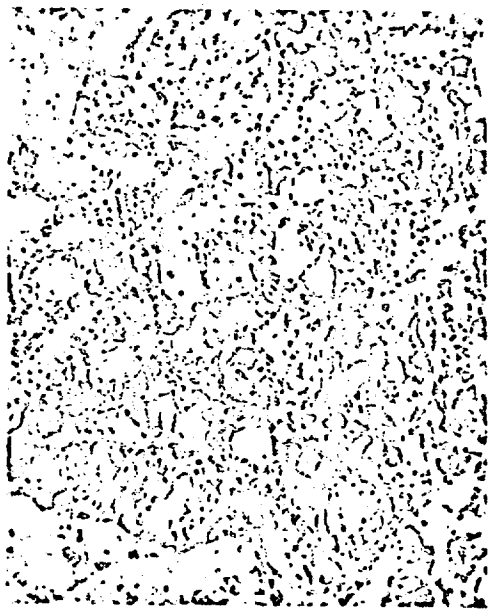

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in the rats were the result of scratching. Not infrequently scratch marks could be seen on the area showing loss of hair, and in a few rats shallow ulcers or scabs were present from time to time. At autopsy the skin appeared to be thin and definitely adherent to the mammary tissue and the vessels immediately beneath the epidermis were engorged. The loss of hair persisted for many months.

The precise aetiology of the tumours is equally mysterious. There are several possibilities and these are not mutually exclusive :

1. Often repeated trauma due to irritation and scratching.
2. Anoxia due to the changes in the vessels.
3. Induction of a tumour in relation to material which cannot be removed from the tissues e.g. tumours induced by plastic films (Oppenheimer, Stout, Oppenheimer and Willhite, 1957).
4. A chronic or repeated stimulus to the production of fibroblasts due to the continued presence of carrageenin acting as a fibroblastic ground substance, or due to the damage which it has caused.
5. The fibrous tissue response to an abnormal ground substance may not be a normal response. For instance Vasiliev (1959) noted that the connective tissue reaction to a pellet containing dimethylbenzanthracene or methylcholanthrene was abnormal—" a distorted fibrogenesis " in which after 3 or 4 months multiple sarcomatous foci appear.

SUMMARY

1. Young female rats were injected into the right flank with a single dose of an acylethyleneimine and into the left flank with a sulphated polygalactose, carrageenin, followed 7 days later by an injection of the acylethyleneimine into the carrageenin induced mass of fibroblasts.

2. Control rats were injected with carrageenin into the left flank and with saline into both flanks.

3. No rats produced sarcomas on the right side, but 11 out of 39 control rats developed a sarcoma on the side injected with carrageenin.

4. 5 out of 23 rats injected with carageenin + caproylethyleneimine and 9 out of 25 rats injected with carageenin + acetylethyleneimine developed sarcomas on the left side, but there was no statistical evidence that the acylethyleneimine had increased the incidence of tumours or shortened the induction period, but in a preliminary experiment using caproylethyleneimine more sarcomata were produced at an earlier date.

5. Carrageenin induced a degeneration of mammary gland epithelium with fibrous tissue replacement and a hyaline thickening of capillary walls. Numerous small nerves were caught in the fibrosis and there was prolonged loss of hair over the injection site.

I wish to thank Dr. F. L. Rose, F.R.S., of Imperial Chemical Industries Ltd., who kindly supplied the capryol and acetylethyleneimines, and Mr. L. Stoloff of the Seaplant Chemical Corporation for the carrageenin. I am greatly indebted to Mr. R. G. Carpenter and Miss A. Young of the Department of Human Ecology, University of Cambridge, for the statistical analysis of data and to Miss N. C. Burr of the Department of Pathology for the care of the animals and for technical assistance.

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THE CARCINOGENIC ACTION OF CARRAGEENIN IN RATS

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ROBERTSON and Schwartz (1953) showed that "carrageenin", (a sulphated polygalactose made from the sea weed, Irish moss, *Chondrus crispus*,) when injected subcutaneously into guinea-pigs would induce the proliferation of fibroblasts and the formation of a connective tissue granuloma. This technique was used to study the biochemistry of collagen formation by Jackson (1956*a* 1956*b*, 1957), Slack (1956, 1957, 1958), Robertson and Hinds (1956), Robertson, Hiwett and Herman (1959). The histology of the lesions produced has been described by Williams (1957) and Benitz and Hall (1959).

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Expected number per 1000 developing sarcomas in 825 days	—	635·9 ± 227·4	536·7 ± 140·0	547·2 ± 136·4	721·5 ± 175·6
Expected number in experiment developing sarcomas in 825 days	—	14·6	14·0	21·3	29·6

NOTE: The expected numbers above refer to the expected numbers per thousand that would develop sarcomas if there were no deaths from other causes.

in mean induction times at 650 days is between group A and the other three groups. The difference between groups A and B is not significant at the 5 per cent level. The difference between groups A and C, and A and D exceed twice their standard errors, but it is not possible to assess the precise significance of their difference owing to uncertainty as to the degrees of freedom that should be attached to the standard errors. Sukhatme's d test (Fisher and Yates, 1953 Table V. 1) indicates, however, that the differences are near the 5 per cent level. Further the larger standard deviation of group A (which is approximately given by $\sqrt{9} \times 20{\cdot}8 = 62{\cdot}4$) compared with the other groups suggests that the form of the distribution of induction times for group A may be different from that of groups B, C and D.

After 825 days, there is no statistically significant difference between the mean induction times for groups B, C and D. If the results from groups A and B are pooled there is no statisticant difference between the mean induction time for this group and the remaining two groups C and D.

Table II shows, assuming no deaths from other causes, the number of rats per thousand that would be expected to have developed tumours after both 650 and 825 days. After 650 days there is no statistically significant difference between groups B, C and D, but there is between these groups and group A, the latter developing more tumours per thousand. After 825 days there is again no statistically significant difference between groups B, C and D.

The figures were computed according to Irwin (1949)

Macroscopic changes induced by carrageenin

A mass was palpable in the region of the injection site for about two weeks. Loss of hair was noticed over the injection site commencing about three weeks after the injection and persisting in many cases for many months. When tumours began the palpable mass usually increased very rapidly in size. In most cases it was necessary to kill the animal within three weeks of first detecting the tumour but sometimes a small mass was felt for some weeks and then suddenly increased rapidly in size. The changes in the mammary tissue at autopsy included a visible increase in the amount of mammary tissue on the left side, a fluid or creamy consistency of the fat, numerous small vessels in the injection area and adherence of the mass to the skin.

Histological appearances of the changes induced by carrageenin

The essential changes induced by carrigeenin can be described under 6 headings.

1. *The early changes.*—These consist of an accumulation of polymorphonuclear leukocytes, macrophages with phagocytosed polysaccharide and fibroblasts. Fig. 1 shows the appearance one week after the injection and shows many macrophages and numerous young fibroblasts. Later mature collagen fibres are in greater evidence. The early changes have been described in detail by Williams (1957) and Benitz and Hall (1959) and need not be further elaborated here.

2. *Degeneration of mammary gland epithelium* was seen in progress over more than two years after the carrageenin injection. Fig. 2 shows the typical appearance of the unaffected mammary tissue on the right side. Note a small cluster of gland alveoli separated by much adipose tissue from its nearest neighbour. Note also the thin walled normal capillaries. Fig. 3 shows that the changes

induced by carrageenin are still progressing 813 days after injection. Note in the centre of the field mammary epithelial cells in process of degeneration. In other parts of the field the empty spaces represent gland alveoli which have degenerated. There is a great increase of the intra-lobular fibrous tissue.

3. *Invasion of degenerated gland alveoli by fibroblasts.*—Fig. 4 shows a good example of this in the lower part of the field; note how the space left by a mammary gland alveolus which has degenerated has now been filled with a group of fibroblasts. A somewhat earlier stage of this process is seen near the middle of the right margin of this picture.

4. *Thickening of the walls of the capillaries and arterioles.*—This change which can be seen in Fig. 3 and 4 is illustrated more clearly in Fig. 5, 6 and 7. Fig. 5 is stained with methyl violet and shows the thick layer of hyaline material surrounding the endothelial lining of the capillary. There are many mast cells present immediately outside this hyaline material. With methyl violet the hyaline material is faintly metachromatic and the mast cells stain deep blue. Fig. 6 is from the same block and is stained with P.A.S. At the bottom of the picture the thickened capillaries and the mast cells are very clear. In the top right hand corner, there are capillaries with walls as much as 4 times as thick as the diameter of the capillary lumen. Fig. 7, stained with haematoxylin and eosin, shows a small arteriole with a greatly thickened wall running in a tortuous spiral diagonally across the field. Other thickened vessels can be easily identified in other parts of this picture.

5. *Involvement of small nerves in the fibrous tissue.*—The fibrous tissue replacement of the mammary tissue appears to enmesh many small nerves. Fig. 8 shows a typical field. Fig. 9 shows four of the changes induced by carrageenin: —the cells lining the mammary gland alveoli have become pale staining, with vacuolated cytoplasm and small pyknotic nuclei; there is definite replacement of gland cells by fibroblasts in the central alveolus; the capillary walls are thickened and a small nerve has been caught in the area of fibrosis.

6. *Sarcomatous changes.*—Zones of precancerous proliferation and early sarcomatous changes were seen in some of the sections. Fig. 10 is from a zone 1 to 2 mm. across which contains many large cells with heavy staining and abnormal nuclear formations. Fig. 11 shows part of an early fibroblastic sarcoma. Most of the large tumours were of this type but some were fibro-sarcomata containing collagen fibres stainable by Van Gieson and some were more anaplastic in type. One typical myxosarcoma was seen.

One rat with a large fibroblastic type sarcoma had bilateral deposits at the lower poles of both kidneys, see Fig. 12 which shows the tumour cells infiltrating between the kidney tubules. A lymphosarcoma of the thymus was also noted in another rat.

DISCUSSION

The original purpose of the experiment was to show whether a single dose of an acylethyleneimine, a simple but short acting carcinogen, would produce a tumour if placed in contact with young, actively growing fibroblasts. The whole basis of the experiment was invalidated by the quite unexpected finding that carrageenin by itself would produce sarcomata. Careful statistical analysis of the results did not show a significant synergistic activity of the single dose of ethyleneimine and the carrageenin.

The long-lasting changes produced by the carrageenin were also quite unexpected. Williams (1957) and Benitz and Hall (1959) described the histological changes produced by carrageenin in the first 28 days following injection. The author has been unable to find any description in the literature of the late effects of injected carrageenin. The degenerative changes in mammary gland epithelium and replacement by fibroblasts are what might have been anticipated but the curious changes in the small vessels were a surprise. The small vessels appear like pipes with thick walls, the hyaline material stains slightly pink with methyl violet, red with periodic-Schiff, eosinophil with haematoxylin and eosin and blue with the picro Mallory stains. Frequently there are many mast cells surrounding these vessels. It is possible that the chronic changes in the mammary gland are due to the interference with its blood supply, but macroscopically the carrageenin-injected tissue appears to be much more vascular than the control gland.

The loss of hair over the injection site is also something of a mystery. It might be due to irritation and scratching by the animal—irritation due to alterations of blood supply or the inclusion of nerve fibres in the mass of fibrous tissue. On the the other hand the hair loss might be due to the interference with the subepidermal layer of elastic tissue and hair follicles. Williams (1957) notes that intradermal injection of carrageenin in the guinea-pig produced after 5 days a degeneration of the collagenous fibres in the dermis, and despite partial remodelling in the later stages the dermis suffered a substantial loss of its collagenous tissue. My clinical impression is that some of the changes in the skin

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EXPLANATION OF PLATES

FIG. 1.—Subcutaneous tissue of a rat 7 days after injection of carrageenin only; note the numerous macrophages and fibroblasts. H. & E. ×570.

FIG. 2.—Control mammary tissue (on right side) 722 days after start of experiment. Note thin walled capillary and much interlobular adipose tissue. Picro Mallory. ×475.

FIG. 3.—Degeneration of mammary gland epithelium with increase of intralobular fibrosis and hyaline thickening of capillary walls seen 454 days after injection of carrageenin + caproylethyleneimine. Picro Mallory. ×360.

FIG. 4.—Degenerated mammary gland epithelium being replaced by fibroblasts, see gland alveolus at bottom centre, also hyaline thickening of capillary walls, 545 days after injection of carrageenin – acetylethyleneimine. Picro Mallory. ×360.

FIG. 5.—Hyaline thickening of capillary walls which are surrounded by mast cells, same rat as Fig. 3, 454 days after injection of carrageenin – caproylethyleneimine. Methyl violet. ×360.

FIG. 6.—From the same rat as Fig. 3 and 5, stained with P.A.S. ×360.

FIG. 7.—Tortuous arterioles and capillaries with hyaline thickening of their walls, 803 days after injection of carrageenin, only. H. & E. ×360.

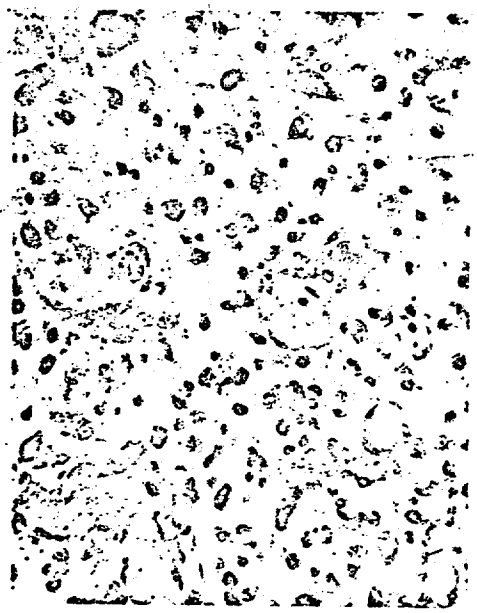
FIG. 8.—A number of fine nerves caught in the fibrous tissue replacing the mammary tissue 813 days after injection of carrageenin only. Picro Mallory. ×360.

FIG. 9.—Typical carrageenin changes—pale vaculated mammary gland epithelium with pyknotic nuclei with replacement by fibroblasts, upper centre; there is a small nerve caught in the process and some hyaline thickening of capillary walls 562 days after injection of carrageenin – acetylethyleneimine. Picro Mallory. ×360.

FIG. 10.—Section from a small zone 1-2 mm. in diameter in which sarcomatous transformation has taken place, 545 days after injection of carrageenin – acetylethyleneimine. H. & E. ×360.

FIG. 11.—Typical fibroblastic type of sarcoma 618 days after carrageenin injection – caproylethyleneimine. The tumour 40 × 15 × 15 mm. in size appeared 28 days previously. H. & E. ×570.

FIG. 12.—Tumour invading right kidney. This is a pleomorphic-cell sarcoma in left flank 722 days after injection of carrageenin + acetylethyleneimine. H. & E. ×360.



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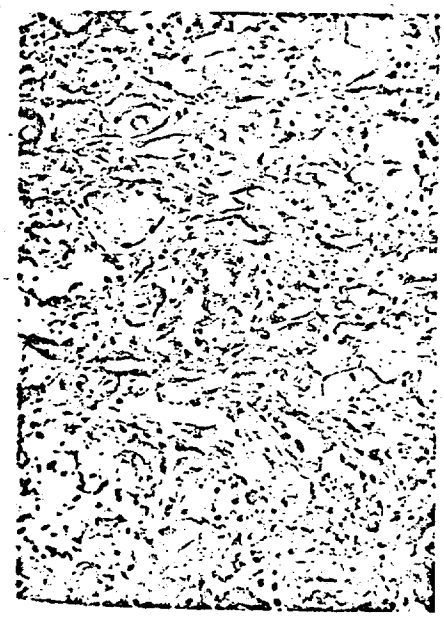

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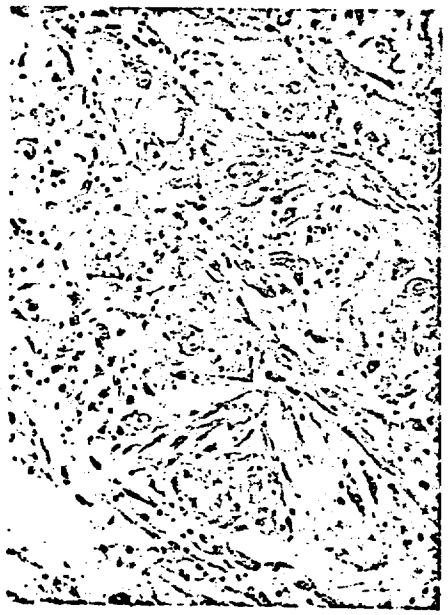

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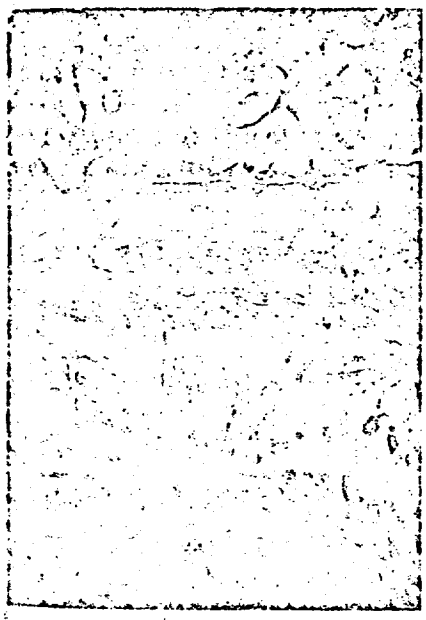

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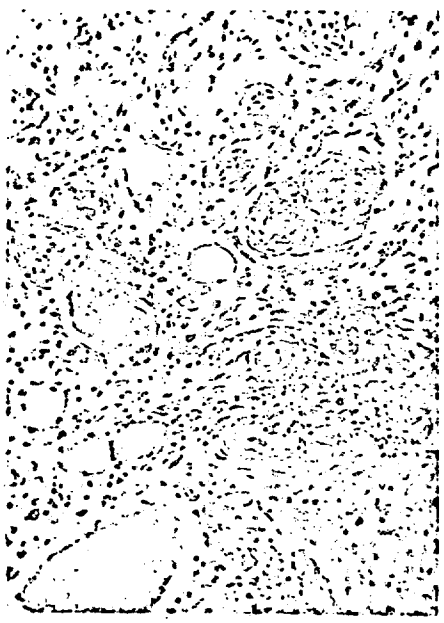

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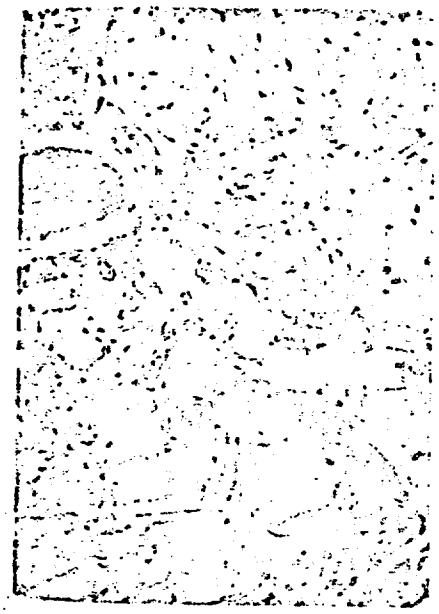

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in the rats were the result of scratching. Not infrequently scratch marks could be seen on the area showing loss of hair, and in a few rats shallow ulcers or scabs were present from time to time. At autopsy the skin appeared to be thin and definitely adherent to the mammary tissue and the vessels immediately beneath the epidermis were engorged. The loss of hair persisted for many months.

The precise aetiology of the tumours is equally mysterious. There are several possibilities and these are not mutually exclusive :

1. Often repeated trauma due to irritation and scratching.
2. Anoxia due to the changes in the vessels.
3. Induction of a tumour in relation to material which cannot be removed from the tissues e.g. tumours induced by plastic films (Oppenheimer, Stout, Oppenheimer and Willhite, 1957).
4. A chronic or repeated stimulus to the production of fibroblasts due to the continued presence of carrageenin acting as a fibroblastic ground substance, or due to the damage which it has caused.
5. The fibrous tissue response to an abnormal ground substance may not be a normal response. For instance Vasiliev (1959) noted that the connective tissue reaction to a pellet containing dimethylbenzanthracene or methylcholanthrene was abnormal—" a distorted fibrogenesis " in which after 3 or 4 months multiple sarcomatous foci appear.

SUMMARY

1. Young female rats were injected into the right flank with a single dose of an acylethyleneimine and into the left flank with a sulphated polygalactose, carrageenin, followed 7 days later by an injection of the acylethyleneimine into the carrageenin induced mass of fibroblasts.

2. Control rats were injected with carrageenin into the left flank and with saline into both flanks.

3. No rats produced sarcomas on the right side, but 11 out of 39 control rats developed a sarcoma on the side injected with carrageenin.

4. 5 out of 23 rats injected with carageenin + caproylethyleneimine and 9 out of 25 rats injected with carageenin + acetylethyleneimine developed sarcomas on the left side, but there was no statistical evidence that the acylethyleneimine had increased the incidence of tumours or shortened the induction period, but in a preliminary experiment using caproylethyleneimine more sarcomata were produced at an earlier date.

5. Carrageenin induced a degeneration of mammary gland epithelium with fibrous tissue replacement and a hyaline thickening of capillary walls. Numerous small nerves were caught in the fibrosis and there was prolonged loss of hair over the injection site.

I wish to thank Dr. F. L. Rose, F.R.S., of Imperial Chemical Industries Ltd., who kindly supplied the capryol and acetylethyleneimines, and Mr. L. Stoloff of the Seaplant Chemical Corporation for the carrageenin. I am greatly indebted to Mr. R. G. Carpenter and Miss A. Young of the Department of Human Ecology, University of Cambridge, for the statistical analysis of data and to Miss N. C. Burr of the Department of Pathology for the care of the animals and for technical assistance.

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Inhibition of Guinea-Pig Complement *in vitro* and *in vivo* by Carrageenin

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Summary. The sulphated polysaccharide carrageenin is a powerful inhibitor of the haemolytic action of guinea-pig serum complement. *In vitro* carrageenin appears to act at a stage prior to the fixation of C1 to sensitized red blood cells. Intravenous administration of carrageenin to guinea-pigs markedly reduces the level of serum complement activity.

INTRODUCTION

The complex of serum proteins collectively known as serum complement still presents many problems. Not least of these is the role played by complement in various cytotoxic effects of antibody, particularly in reactions of hypersensitivity. Considerable progress in this direction has already been made, particularly by Osler and his collaborators (Osler, 1961; Osler, Randall, Hill and Ovary, 1959) but a definite conclusion is still elusive. A highly active inhibitor of complement which exhibited its action *in vivo* without producing other untoward effects should be of great value in unravelling this problem. None of the known inhibitors is suitable. Zymosan has been used in rats by Osler *et al.* (1959), but since it is known to form anaphylatoxin its effects on hypersensitivity may be complicated. Furthermore, it is insoluble, thus limiting the dose that can be given intravenously. Formaldehyde and primary amines, such as hydrazine, inhibit complement, but are too toxic for systemic use. Salicylaldoxime (Mills and Levine, 1959) and phloretin (Rodriguez and Osler, 1958, 1960) are too weakly active. The present experiments suggest that a suitable inhibitor has been found in carrageenin, a sulphated polysaccharide obtained from *Chondrus* spp.

MATERIALS AND METHODS

Buffer

This was barbitumate buffer, pH 7·3, ionic strength 0·147 prepared as described by Mayer (1961). Unless otherwise stated, it contained 0·00015 M Ca^{++} and 0·0005 M Mg^{++}.

Complement

Guinea-pigs were bled from the heart under ether anaesthesia; the blood was allowed to clot and the serum harvested after centrifugation. The number of 50 per cent units of haemolytic complement (CH_{50}) per ml was estimated from the optical density at 541 mμ using a Unicam SP600 spectrophotometer. Only sera with complement titres between 250 and 300 CH_{50} ml. were used. The sera were stored at −20° for not more than 1 week, during which period the complement titre remained constant.

Sheep Erythrocytes

Sheep blood in Alsever's solution was purchased from Evans Medical Ltd., Liverpool. Before use the cells were washed three times with barbiturate buffer and standardized spectrophotometrically to contain 10^9 cells/ml. Sensitization was effected by mixing the cells with an equal volume of haemolysin at its optimal concentration. Sensitized cells are designated by the symbol EA.

Haemolysin

Rabbits were immunized with boiled stromata from sheep red blood cells as described by Mayer (1961).

Intermediate Complexes of Cells and Complement Components

(a) Sensitized cells containing the first component of complement ($EAC_1^{\prime}$) were prepared by the method of Klein (1960). This method depends upon the destruction of C_2 on treating whole serum with ether. Suspensions of EA were incubated with the ether-treated serum in buffer containing 0·001 M Ca^{++} but no Mg^{++}. Since $C_2^{\prime}$ will not react with EA in the absence of $C_2^{\prime}$, the cells remain at the stage $EAC_1^{\prime}$. The cells were always used on the day of preparation. Immediately before use they were centrifuged, washed and suspended in buffer containing 0·00015 M Ca^{++} and 0·0005 M Mg^{++} and standardized to contain 5×10^8 cells/ml.

(b) Sensitized cells containing the first and fourth components of complement ($EAC_{1,4}$) were prepared according to Mayer (1961). Satisfactory preparations were obtained by adding 0·5 ml. of guinea-pig serum to optimally sensitized erythroctyes (10^9/ml.) in buffer containing 0·001 M Ca^{++}. The mixture was allowed to react at 0° for 30 minutes with occasional agitation. After washing and incubating for 90 minutes at 37° (to allow the $EAC_{1,4,2}^{\prime}$ to revert to $EAC_{1,4}$) the cells were again washed, suspended in buffer with optimal Ca^{++} and Mg^{++} and standardized to contain 5×10^8 cells/ml.

(c) Sensitized cells containing the first, fourth and second components of complement ($EAC_{1,4,2}$) were prepared by the method of Rodriguez and Osler (1960). Two ml. volumes of complement were treated with 0·1 ml. volumes of 1:20 to 1:40 formalin for 40 minutes at 37°. Ten ml. amounts of EA (10^9/ml.) were then incubated for 20 minutes at 30°, rapidly chilled by addition of cold buffer containing 0·01 M EDTA, centrifuged and suspended in EDTA buffer to contain 5×10^8 cells/ml.

Carrageenin

Two types were used and appeared to be equally effective. Sea-Kem type 402 was obtained in 1958 from Seaplant Chemical Corporation, New Bedford, Mass., Sea-Kem type 21 was kindly donated by Marine Colloids, Inc., 24 State St., New York, who are the successors to Seaplant Chemical Corporation.

Solutions were made by dissolving the carrageenin in boiling saline and allowing it to cool to room temperature. At ice-bath temperature there was a tendency for the carrageenin to come out of solution.

RESULTS

The studies on carrageenin started with attempts to relate the formation of anaphylatoxin to the fixation of complement in pursuance of the work of Osler and his colleagues

For this purpose various polysaccharides were tested for their abilities to form anaphylatoxin from guinea-pig serum and to inhibit guinea-pig complement. These two effects appeared to be correlated except for carrageenin, which inhibited complement but did not form anaphylatoxin (Table 1). Further experiments showed that this inhibition was present at very low concentrations of carrageenin (Table 2) and was proportional to the initial concentration of complement (Table 3).

TABLE 1

EFFECTS OF POLYSACCHARIDES ON SERUM COMPLEMENT AND ON FORMATION OF ANAPHYLATOXIN FROM GUINEA-PIG SERUM

Polysaccharide	*Anaphylatoxin formation*	*Anticomplementary effect*
Agar	+	±
Inulin	+	+
Dextran	+	±
Starch	−	−
Zymosan	+	++
Carrageenin	−	+++

TABLE 2

INHIBITION OF GUINEA-PIG COMPLEMENT BY CARRAGEENIN

Whole serum incubated for 30 minutes with equal volumes of carrageenin

Final concentration of carrageenin (mg./ml.)	*Residual C′ activity ($C'H_{50}$/ml.)*
0	210
0·015	80
0·03	<10
0·06	<8
0·12	<3
0·25	0

Since other polysaccharides appear to react preferentially with C'_3, an attempt was made to demonstrate an inhibitory effect of carrageenin on the reaction between cells in the form $EAC'_{1,4,2}$ and C′ in the presence of EDTA (C'_3).

Guinea-pig serum was incubated at 37° for 30 minutes with an equal volume of carrageenin solution (0·2 mg./ml.) in EDTA buffer. A sample of control serum was diluted with an equal volume of EDTA buffer at the same time. Mixtures containing 0·5 ml. of $EAC'_{1,4,2}$ cells ($1{\cdot}5 \times 10^8$/ml.) and 2 ml. of dilutions in EDTA buffer of either carrageenin-treated or control serum were then incubated for 90 minutes at 37°. Five ml. of ice-cold EDTA buffer was added to each tube, the tubes centrifuged and the optical density of the supernatant measured at 412 mμ because of the low concentration of the cells. The results in Table 4 show that carrageenin had not destroyed C'_3 nor had it affected the ability of C'_3 to react with $EAC'_{1,4,2}$.

Since carrageenin is a sulphated polysaccharide it appeared possible that it was sequestering calcium and magnesium and thus hindering fixation of C'_1, C'_4 and C'_2. An

attempt was made to reverse the inhibitory effect by adding excess calcium and magnesium. Tubes were set up containing 1 ml. of complement (20 $C'H_{50}$), 1 ml. of carrageenin solution, 1 ml. of EA (5×10^8 cells/ml.) and 4·5 ml. of buffer containing one of the following: (a) 0·0005 M Mg^{++} and 0·00015 M Ca^{++}; (b) 0·0005 M Mg^{++} and 0·0015 M Ca^{++}; (c) 0·005 M Mg^{++} and 0·00015 M Ca^{++}; and (d) 0·005 M Mg^{++} and 0·0015 M Ca^{++}.

TABLE 3

In vitro INHIBITION OF COMPLEMENT BY CARRAGEENIN: DOSE RESPONSE

$C'H_{50}$	*Carrageenin (μg.)*	*Per cent lysis*
5	3·3	0
5	1·7	1·4
5	0·99	1·4
5	0·69	2·8
5	0·55	12·3
5	0·39	65·8
5	0·23	99·0
5	0·12	100·0
10	6·4	0
10	3·2	4·0
10	1·9	6·0
10	1·3	10·0
10	1·1	21·4
10	0·75	65·5
10	0·45	97·3
10	0·23	100·0
20	13·0	2·6
20	6·5	6·5
20	3·9	8·5
20	2·7	22·3
20	2·2	59·5
20	1·5	100·0
20	0·92	100·0
20	0·46	100·0

TABLE 4

EFFECT OF CARRAGEENIN ON REACTION OF $EAC_{1,4,2}$ WITH C'_3

Dilution of serum added to $EAC_{1,4,2}$	*Per cent lysis*	
	With carrageenin (carrageenin serum 0·1 mg./ml.)	*Without carrageenin*
1 : 10	100	100
1 : 20	100	99
1 : 40	76	75
1 : 80	41	40
1 : 160	16	13

The degree of inhibition of C′ by carrageenin was the same in tubes containing normal Ca^{++} and Mg^{++} and in those containing ten times normal Ca^{++} and normal Mg^{++}. Carrageenin appeared to be more active in the presence of excess Mg^{++} but this is probably only a reflection of the known inhibitory effect of excess Mg^{++} (Table 5).

An attempt was made to identify the carrageenin-sensitive component(s) by using classical complement reagents, each of which is deficient in a single component. The reaction mixtures contained 5 $C'H_{50}$ of complement, 1 μg. of carrageenin and 5×10^8 EA/ml. in a total volume of 6·5 ml. of buffer. To these, 1 ml. amounts of various reagents were added and the mixtures incubated for 90 minutes at 37° and the amount of haemolysis measured. Carrageenin-induced inhibition was reversed not only by whole serum

TABLE 5

EFFECT OF ADDED Ca^{++} AND Mg^{++} ON THE INHIBITORY ACTION OF CARRAGEENIN

Concentration of carrageenin (mg./ml.)	*Per cent lysis*			
	Normal buffer	0·001 M Ca^{++}	0·005 M Mg^{++}	0·0015 M Ca^{++} + 0·005 M Mg^{++}
0·02	9·6	10·1	10·4	10·1
0·01	13·2	13·9	13·9	11·0
0·005	36·0	48·0	13·7	12·5
0·0025	68·0	85·5	33·0	18·3
0·00125	100·0	100·0	100·0	82·0
0·0006	100·0	100·0	100·0	100·0

TABLE 6

REVERSAL OF CARRAGEENIN INHIBITION BY VARIOUS COMPLEMENT REAGENTS

Complement reagents	*Per cent lysis*	
	With 1 μg. carrageenin	*Without carrageenin*
None	11·2	100·0
1 : 10 whole C′	100·0	100·0
1 : 25 whole C′	100·0	100·0
1 : 10 R_1	100·0	100·0
1 : 10 R_2	100·0	100·0
1 : 10 R_3	100·0	100·0
1 : 10 R_4	100·0	100·0

but also by all the reagents (Table 6). Since the reagents R_1, R_2, R_3 and R_4 contain none of the known complement components in common, it appeared probable that carrageenin was destroying some other component. This impression was strengthened by the use of sensitized cells containing various intermediate complexes of complement. Tubes containing 30 $C'H_{50}$ of complement and 50 μg. of carrageenin in a total volume of 2 ml. were incubated for 10 minutes at 37°. A duplicate set of tubes containing complement but no carrageenin was similarly prepared. To each tube, 4·5 ml. of buffer and 1 ml. containing 5×10^3 of one of the following was added: EA, EAC_1, $EAC_{1,4}$ or $EAC_{1,4,2}$. The two tubes which were to receive $EAC_{1,4,2}$ contained 0·01 M EDTA. In all other tubes, buffer containing optimal Ca^{++} and Mg^{++} was used. The amount of haemolysis was determined

TABLE 7

EFFECT OF CARRAGEENIN ON INTERMEDIATE PRODUCTS OF SENSITIZED CELLS AND COMPLEMENT

Intermediate product	*Per cent lysis*	
	With 50 μg. carrageenin	*Without carrageenin*
EA	15·3	100·0
EAC_1'	96·0	98·0
$EAC_{1,4}'$	79·0	83·5
$EAC'_{1,4,2}$	89·0	93·0

Each tube contained 30 CH_{50}. $EAC_{1,4,2}'$ in buffer with 0·01 M EDTA. Other products in buffer with 0·0005 M Mg^{++} and 0·0015 M Ca^{++}.

after incubation for a further 60 minutes (Table 7). It is apparent from these results that cells which have reached the stage EAC_1' or any of the known subsequent stages will still proceed to lysis in the presence of complement and carrageenin, even though the latter is still able to inhibit lysis of EA by the same amount of C'. The first component itself is not inhibited since the reagent deficient in C_1' (R_1) will reverse carrageenin-induced inhibition. Carrageenin must therefore act at some stage prior to the fixation of C_1'.

HEAT STABILITY OF 'CARRAGEENIN-SENSITIVE COMPONENT'

The above results suggest that carrageenin could exert its effect by inhibiting a complement component which reacts with sensitized cells before C_1'. An attempt was made to determine the heat stability of this postulated component. Five ml. of whole serum was incubated for 30 minutes with 5 ml. of carrageenin solution (0·1 mg./ml.) Reaction mixtures were then prepared to contain: 1 : 5 carrageenin-treated serum (1 ml.); EA (5×10^8 cells in 1 ml.); buffer (4·5 ml.) and 1 : 5 fresh serum or serum heated for various periods at 56° (1 ml.). The results in Table 8 show that inhibition was completely reversed by unheated serum but that reversal became progressively less effective as the serum was heated. There was, however, some reversing activity still present after 20 minutes heating.

TABLE 8

HEAT STABILITY OF THE CARRAGEENIN-SENSITIVE C' COMPONENT

Addition to mixture of sensitized cells plus carrageenin-treated serum	*Per cent lysis*
None	6·4
1 ml. 1 : 5 serum heated at 56° for	
5 minutes	90·0
10 minutes	67·1
20 minutes	38·5

Carrageenin-treated serum: 5 ml. of serum incubated with 5 ml. of carrageenin 0·1 mg./ml. for 30 minutes at 37°. Whole complement activity in serum was destroyed completely after 10 minutes at 56°.

This is in contrast to the haemolytic activity of this sample which was completely destroyed after 10 minutes at 56°.

INHIBITION OF COMPLEMENT BY SYSTEMIC CARRAGEENIN

Guinea-pigs given intravenous injections of up to 5 mg./kg. of carrageenin exhibit no symptoms apart from a slightly prolonged bleeding time at the site of the intravenous injection. Serum from these animals, however, shows a marked depletion in the level of circulating complement (Table 9). Animals receiving doses as low as 1 mg./kg. had only

TABLE 9

In vivo INHIBITION OF COMPLEMENT BY CARRAGEENIN

Dose of carrageenin (mg./kg. i.v.)	*$C'H_{50}$/ml.*
5·0	15, 24, 10, 8
2·5	18, 21, 16, 20
1·0	100, 125, 95, 90
Controls	200, 226, 264, 200

Animals bled 10 minutes after injection.

TABLE 10

In vivo INHIBITION OF COMPLEMENT BY CARRAGEENIN

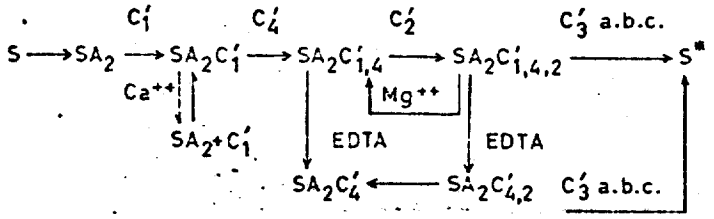
Time after injection of 5 mg./kg. i.v. carrageenin (min.)	*$C'H_{50}$/ml.*
10	2
20	3
30	1
40	10
50	80
Controls	200

Two animals per group.

about half the normal levels. After a dose of 5 mg./kg. the complement levels remained very low for about 30 minutes after injection and then started to recover (Table 10). Intraperitoneally administered carrageenin had no effect on serum complement in doses up to 20 mg./kg.

DISCUSSION

The most recent, and most complete scheme for the sequence of reactions of complement components with sensitized cells is that of Borsos and Mayer (1961).



[illegible] for an antigenic site on the surface of the erythrocyte, A_2 for two molecules of antibody, C_1^1, etc., for the various components of complement, and C_3^1 a.b.c. for the three factors comprising the third component of complement. Cells possessing one site in the form S* will lyse spontaneously. The intermediate products, at a cellular level, are usually designated by the symbols EA, $EAC_{1,4}^1$, etc.

Consideration of this scheme reveals multiple steps at which an inhibitor could act. However, cells in the form EAC_1^1 (or $EAC_{1,4}^1$ or $EAC_{1,4,2}^1$) will still progress to lysis in the presence of a concentration of carrageenin which prevents the lysis of EA by whole C'.

The carrageenin-sensitive step therefore precedes the formation of EAC_1^1. Calcium is required for the formation of EAC_1^1; excess calcium does not, however, prevent the inhibitory action of carrageenin. Carrageenin therefore either prevents the fixation of C_1^1 directly or acts on a component of complement which precedes the fixation of C_1^1.

Müller-Eberhard and Kunkel (1961) have described a component of human complement, designated 11S, which reacts with EA before C_1^1 and in the presence of EDTA. We are at present attempting to identify this component with the carrageenin-sensitive component of guinea-pig serum. Both components have only limited heat stability.

Anti-complementary effects of sulphonated esters of cellulose have been described by Mutsaars and Lison (1948) who concluded that C_1^1, C_2^1 and C_3^1 were inhibited, but not C_4^1. The experiments, however, were not very well controlled and some doubt on their conclusions arises from the incorrect assumption that C_4^1 is the first component to react with the immune complex.

Carrageenin appears to be the first inhibitor of complement which can be used in living guinea-pigs without producing obvious side effects, apart from a slight anticoagulant action. It should therefore prove useful for elucidating the role of complement in hypersensitivity reactions. Such experiments are in progress.

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Inhibition of Complement by Carrageenin: Mode of Action, Effect on Allergic Reactions and on Complement of Various Species

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Summary. Carrageenin prevents the reaction of guinea-pig C′ with sensitized red blood cells, probably by a direct inactivation of the C'_1. The anti-complementary effect of carrageenin is antagonized by protamine sulphate. Marked reduction of circulating C′ activity in guinea-pigs resulting from intravenous injection of carrageenin, does not alter their susceptibility to acute or protracted anaphylactic shock. The C′ activities of human, guinea-pig, rabbit, rat and monkey sera are all inhibited *in vitro* by carrageenin.

INTRODUCTION

The haemolytic activity of guinea-pig complement is inhibited by very small amounts of the sulphated polysaccharide carrageenin, both when the carrageenin is added to the complement *in vitro* and when it is given intravenously to the animal before it is bled. Carrageenin appears to act at a stage prior to the fixation of the first component of complement (C'_1) to the sensitized red blood cell (EA) since cells in the forms EAC'_1, $EAC'_{1,4}$ and $EAC'_{1,4,2}$ still lyse in the presence of their appropriate reagents, irrespective of the presence of carrageenin (Davies, 1963). The present experiments were designed to elucidate further the mode of action of carrageenin, to observe the effect of carrageenin treatment on anaphylactic reactions in guinea-pigs and to test the effect of carrageenin on the complement activity of sera from species other than the guinea-pig.

MATERIALS AND METHODS

Buffer

Barbiturate buffer, pH 7·3, ionic strength 0·147, was prepared as described by Mayer (1961). Unless otherwise stated, it contains 0·00015 M Ca^{--} and 0·0005 M Mg^{--}. Ethylene diamine tetra-acetic acid, when required was used at a concentration of 0·01 M, in which case calcium and magnesium were omitted from the buffer.

Complement

Guinea-pigs, mice and rats were bled from the heart; monkeys and rabbits were bled from a vein, all under ether anaesthesia. Human blood was taken from a vein, without anaesthesia. Complement was estimated in terms of the number of 50 per cent haemolytic units ($C'H_{50}$) per ml., as described by Mayer (1961). Conditions optimal for the estimation of guinea-pig complement were also used for mouse, rabbit and monkey serum. Optimal conditions for the estimation of rat complement are slightly different (Osler, Hawrisiak,

Ovary, Siqueira and Bier, 1957). They involve the use of a higher concentration of haemolysin and the omission of calcium from the buffer. All estimations were done using a total volume of 7·5 ml., including 1 ml. of sensitized red blood cells (5×10^3). Optical densities were read at 541 mμ using a Unicam SP 600 spectrophotometer.

Sheep Erythrocytes

Sheep blood in Alsever's solution was purchased from Evans Medical Limited, Liverpool. Before use the cells were washed three times with barbiturate buffer and standardized spectrophotometrically to contain 10^9 cells/ml. Sensitization was effected by mixing these cells with an equal volume of haemolysin at its optimal concentration.

Haemolysin

Rabbits were immunized with boiled stromata from sheep red blood cells as described by Mayer (1961).

This haemolysin when added to a suspension of sheep erythrocytes, produced cells in the form EA. For the production of the intermediate complex EAC'_1, a sample of haemolysin was similarly prepared but was not heated before use.

Preparation of EAC'_1

Washed sheep erythrocytes (10^9 cells/ml.) were incubated for 20 minutes at 37° with an equal volume of unheated haemolysin, diluted 1/100 to 1/250 with buffer. These cells lysed in the presence of a reagent deficient in C'_1 (R1) (De Looze, Ransby and Leon, 1962). It should be noted that the C'_1 in this preparation is derived from the unheated rabbit haemolysin.

Reagent Deficient in C'_1 (R1)

This was prepared from guinea-pig serum according to Mayer (1961) and consisted of equal parts of euglobulin (prepared by dialysis and diluted 1/10) and guinea-pig serum heated at 56° for 20 minutes and diluted 1/10.

Carrageenin

Carrageenin, type 21, was kindly donated by Marine Colloids, Inc., 24 State Street, New York. Solutions were made by dissolving the carrageenin in boiling saline and allowing it to cool to room temperature.

Anaphylactic Shock in Guinea-Pigs

Guinea-pigs were sensitized by intraperitoneal injection of alum-precipitated, crystallized egg albumin (2·5 ml. of 2 per cent suspension) and used for experiments 3–4 weeks later. Acute anaphylactic shock was produced by the intravenous injection of crystallized egg albumin (0·2 ml. per 100 g. of a 0·2 per cent solution in saline). Protracted anaphylactic shock (Stone, 1959) was produced by subcutaneous injection of crystallized egg-albumin (0·4 ml. per 100 g. of a 10 per cent solution) in animals which had been dosed with mepyramine (2·5 mg. per 100 g. subcutaneously) 15 minutes before.

Permeability Factor

The permeability factor (PF/P) was prepared and tested by the method of Davies and Lowe (1960) by adding a pre-formed antigen–antibody complex (egg-albumin/rabbit

anti-egg albumin serum) to fresh guinea-pig serum and injecting the mixture into the skin of guinea-pigs previously injected intravenously with pontamine sky blue.

RESULTS

MODE OF ACTION OF CARRAGEENIN

Since cells in the form of the intermediate complex, EAC'_1, still progressed to lysis in the presence of C′ and a concentration of carrageenin which prevented lysis of EA by C′, it was suggested previously that carrageenin prevents the fixation of C'_1 to the sensitized cell (Davies, 1963).

The intermediate complex EAC'_1 can be prepared by allowing red blood cells to react with unheated haemolysin prepared in rabbits (De Looze *et al.*, 1962). Various concentrations (up to 100 μg./ml.) of carrageenin, in 1 ml. of buffer, were incubated with red blood cell suspension (0·5 ml.) and unheated haemolysin (0·5 ml. of 1/250) for 20 minutes at 37° before the addition of R1 (1 ml.) and buffer (4·5 ml.). A second set of tubes was prepared in which red blood cell suspension and unheated haemolysin were incubated together for 20 minutes before the addition of R1 and carrageenin. Both sets of tubes showed complete lysis during subsequent incubation at 37°. This result suggested the unlikely conclusion that carrageenin acted neither before nor after the fixation of C'_1. However, the haemolysin had been added to the cells a minute or so before the carrageenin and there was a possibility that the cells were already in the form EAC'_1 when the carrageenin was added. Unheated haemolysin (0·5 ml. of 1/250) was therefore incubated for 10 minutes with carrageenin (50 μg. in 1 ml.) before the addition of red blood cell suspension (0·5 ml.). Incubation was continued for a further 20 minutes and then R1 (1 ml.) and buffer (4·5 ml.) were added and incubation continued for a further 60 minutes. Similarly, carrageenin was incubated for 10 minutes with cells before the addition of haemolysin and, later, of R1 and buffer. There was no lysis in either of these tubes, indicating that carrageenin had prevented the formation of EAC'_1. This result also showed that, under these conditions, the formation of EAC'_1 was very rapid, as was the reaction between C′ and carrageenin. The following experiments supported these conclusions.

Sensitized cells were cooled to 0° and 1 ml. of the suspension added to 1 ml. of cooled C′ (1 : 100). The mixture was incubated at 0° for 10 minutes. Buffer (4·5 ml.) and carrageenin (5 μg. in 1 ml.) were then added. To a second tube, similarly prepared, buffer (5·5 ml.) alone was added. Both tubes were then incubated for 60 minutes at 37°. In a separate experiment, a series of tubes was prepared in which the various components of the reaction (EA, carrageenin, C′, and buffer) were mixed in different orders, at 30-second intervals at room temperature and then incubated for 60 minutes at 37°. The results showed (Table 1) that whereas cells incubated with C′ at 0° before the addition of carrageenin still progressed to lysis, there was little or no lysis when carrageenin was present before C′. Cells incubated at 0° with C'_1 will be in various forms (EA, EAC'_1, $EAC'_{1,4}$, etc.) but since the earlier experiments showed that the lysis of EAC'_1 is not inhibited by carrageenin the only possible explanations of the failure of cells to lyse is that the formation of EAC'_1 is prevented either by destruction of a C'_1 component or by a protective effect on the cell.

Müller-Eberhard and Kunkel (1961) and Müller-Eberhard (1961) have described a component of human complement, designated 11S component, which reacts with sensitized cells before C'_1 and in the absence of calcium. Its activity was lost upon heating at

56° for 45 minutes. Lepow, Naff, Todd, Pensky and Hinz (1963) identified this component with their C'_{1q}. Davies (1963) showed that the carrageenin-sensitive component in guinea-pig serum was not completely destroyed by heating at 56° for 20 minutes although haemolytic activity was destroyed by heating the serum for 10 minutes.

Because of this apparent similarity in heat-lability of C'_{1q} and the carrageenin-sensitive component and also because carrageenin appeared to act on a C'_1 component, it appeared that the two might be identical.

TABLE 1

INHIBITORY EFFECT OF CARRAGEENIN ON THE FORMATION OF EAC'_1

(a) EAC'_1 formation at 0°

	Lysis (%)
EA + C′ incubated at 0° before addition of carrageenin	83
EA + C′ incubated at 0° before addition of buffer	100

(b) Effect of mixing component in different orders

Order of mixing	*Lysis* (%)
EA; carrageenin; C′; buffer	0
EA; C′; buffer; carrageenin	50
EA; carrageenin; buffer, C′	5
EA; C′; buffer	100
EA; buffer; C′	100

On the assumption, as yet unproven, that the C'_1 complex of guinea-pig serum is similar to that of man, and taking advantage of the finding that C'_{1q} (11S) reacts with EA in the absence of calcium, an attempt was made to prepare the intermediate complex EAC'_{1q} from guinea-pig C′. It was argued that, if carrageenin inhibits C'_{1q}, then cells already in this form should lyse unhindered in the presence of C′ previously incubated with carrageenin.

At 0°, EA (4 ml.) were mixed with C′ (4 ml. of 1/10) and 22 ml. of buffer containing 0·01 M EDTA. After 10 minutes incubation at 0° the cells were washed once with EDTA buffer and three times with buffer containing calcium and magnesium and finally suspended in 4 ml. of this buffer. Another 4 ml. of EA were similarly treated, but the C′ was omitted and 4 ml. of EDTA buffer used in its place. The first lot of cells are referred to as 'postulated EAC'_{1q}'. To prepare the 'postulated $R1_q$', C′ (5 ml. of 1/10) was incubated with carrageenin (5 ml. of 25 μg./ml.) for 45 minutes at 37°. A 1 ml. sample of this mixture produced 9 per cent haemolysis when incubated with EA (1 ml.) and buffer (5·5 ml.). In order to neutralize excess carrageenin, protamine sulphate (500 μg. in 1 ml.) (see below) was added to the remainder and, after incubation for 5 minutes, 1 ml. of the mixture was added to EA (1 ml.) and buffer (5·5 ml.), to test for completeness of inactivation, and a further 1 ml. was added to EA (1 ml.), C′ (1 ml. of 1/200), and buffer (4·5 ml.) to test for anti-complementary activity. There was 26 per cent lysis in the first tube and complete lysis in the second, indicating almost complete destruction of complement activity during the preliminary incubation of C′ with carrageenin and little or no anti-complementary effect of the final reagent. One ml. amounts of 'postulated EAC'_{1q}', or control cells,

were then added to 1 ml. amounts of either 'postulated $R1_q$' or C′ (1/100). Buffer was added to a total volume of 7·5 ml. and the degree of haemolysis measured after incubation for 1 hour at 37°. Both kinds of cells showed complete lysis in the presence of C′ but only about 30 per cent lysis in the presence of 'postulated $R1_q$' (Table 2). It therefore seemed that if the cells were in fact in the form $EAC'1_q$ then their subsequent reaction with other components was blocked by carrageenin.

TABLE 2

EFFECT OF CARRAGEENIN ON 'POSTULATED C'_{1q}'

Reaction mixture	*Lysis* (%)
'EAC'_{1q}' + '$R1_q$'	32
'EAC'_{1q}' + C′ (1/100)	100
EA + '$R1_q$'	37
EA + C′ (1/100)	100

'EAC'_{1q}' = EA incubated with C′ in the presence of EDTA.
'$R1_q$' = C′ incubated with carrageenin and excess carrageenin neutralized by the addition of protamine sulphate.

REVERSAL OF THE ANTI-COMPLEMENTARY EFFECT OF CARRAGEENIN BY PROTAMINE SULPHATE

In order to study the reaction between complement and carrageenin it was necessary to find some means of neutralizing the effect of carrageenin.

Mutsaars and Lison (1948) showed that the anti-complementary effect of sulphated esters of polysaccharides was antagonized by basic dyes. Since the presence of basic dyes

TABLE 3

ANTAGONISM OF THE ANTI-COMPLEMENTARY EFFECT OF CARRAGEENIN BY PROTAMINE SULPHATE

Carrageenin (μg.)	*Protamine sulphate* (μg.)	*Lysis* (%)
10	25	100
10	12·5	14
10	0	7
0	100	100
0	0	100

1 ml. of guinea-pig C′ (1/100) in each tube.

was likely to interfere with the colorimetric estimation of haemoglobin, the basic protein protamine was tested for its ability to neutralize the effect of carrageenin. A concentration of 25 μg. per ml. of protamine sulphate antagonized the anti-complementary activity of 10 μg. per ml. of carrageenin (Table 3). The amount of protamine sulphate that could be used in experiments of this type was limited by the anti-complementary effect of protamine itself, and this, in turn, was antagonized by carrageenin (Table 4).

Antagonism of carrageenin activity by protamine enabled a study to be made of the rate at which complement activity was destroyed by carrageenin and, incidentally, to obtain evidence that carrageenin acted directly on some part of complement, rather than on the sensitized cell itself or on some intermediate complex of components of complement

with the sensitized cell. Complement (1 ml. of 1/100) and buffer (3·5 ml.) were pipetted into a series of tubes. At timed intervals, carrageenin solution (10 μg. in 1 ml.) was added and, at various intervals thereafter, protamine sulphate (50 μg. in 1 ml.) and EA suspension (1 ml.) were added and the tubes incubated for a further 60 minutes at 37°. The

TABLE 4

MUTUAL ANTAGONISM OF ANTI-COMPLEMENTARY EFFECTS BY CARRAGEENIN AND PROTAMINE SULPHATE

Carrageenin (μg.)	*Lysis* (%)					
	No protamine	12·5 μg. *protamine*	25 μg. *protamine*	50 μg. *protamine*	100 μg. *protamine*	200 μg. *protamine*
0	82	75	67	37	9	0
1	0·7	73	75	60	8	0
2	0·6	74	75		9	0
4	1·7	76	81	63	14	0
8	0	3	76	75	22	0
6	0	0	3	80	46	0

1 ml. of guinea-pig C′ (1/200) in each tube.

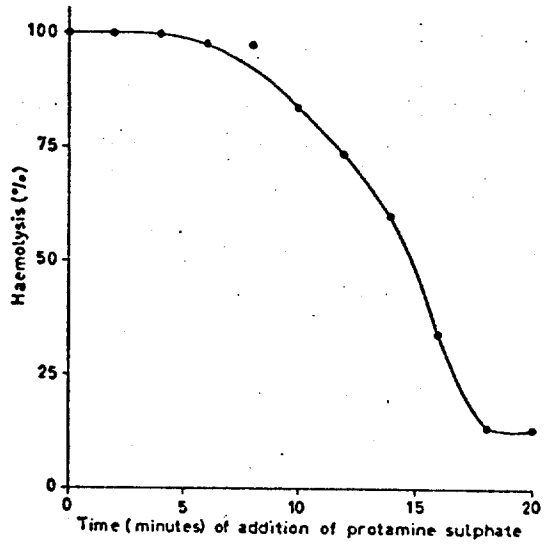

FIG. 1. The rate of destruction of C′ by carrageenin: Complement (1 ml. of 1/100) incubated with carrageenin (10 μg. in 1 ml.). Protamine sulphate (50 μg. in 1 ml.) and EA added at various intervals. Haemolysis measured after 1 hour at 37°.

amount of haemolysis in each tube was plotted against the period of contact between C′ and carrageenin. Under these conditions, complement activity was almost completely destroyed in about 20 minutes (Fig. 1).

EFFECT OF CARRAGEENIN ON ALLERGIC REACTIONS

Since intravenously-administered carrageenin inhibited the haemolytic complement activity in the serum of dosed guinea-pigs (Davies, 1963) it was of obvious interest to study the effect of such dosing on allergic reactions in this species.

Sensitized guinea-pigs were subjected to acute and protracted anaphylactic shock. Neither condition was affected by a dose of carrageenin (5 mg./kg.) given intravenously 10 minutes before the injection of antigen (Table 5). This amount of carrageenin reduced the C′ activity to less than 5 $C'H_{50}$/ml.

Davies and Lowe (1960) described a permeability factor formed by adding antigen–antibody complexes to guinea-pig serum. This factor, which they called PF/P appeared to require the presence of serum complement for its formation (Davies and Lowe, 1962). Its formation was not, however, inhibited by carrageenin.

TABLE 5

LACK OF EFFECT OF INTRAVENOUSLY ADMINISTERED CARRAGEENIN ON ANAPHYLAXIS IN GUINEA-PIGS

(a) Anaphylaxis		(b) Protracted anaphylaxis	
Survival time (minutes)		*Survival time (minutes)*	
Carrageenin (5 mg./kg. i.v.)	*Controls*	*Carrageenin (5 mg./kg. i.v.)*	*Controls*
3	4	42	45
4	3	57	29
2	2	31	Survivor
5	5	50	18
2	3		

EFFECT OF CARRAGEENIN ON THE COMPLEMENT OF SPECIES OTHER THAN THE GUINEA-PIG

Carrageenin inhibited the haemolytic activity of the complement of all five species tested. Its activity was not related to the C′ titre of the serum since it was equally active against human, monkey and guinea-pig C′. Both rat and rabbit C′ were very sensitive to the inhibitory effect when they were tested under conditions suitable for the assay of guinea-pig C′ (Table 6).

TABLE 6

INHIBITION OF COMPLEMENT BY CARRAGEENIN *in vitro*

Species	$C'H_{50}$ *(ml.)*	*Haemolysis (%)*								
		200*	100	50	25	12·5	6·25	3·12	1·56	0·78
Guinea-pig	250	0	0	1·0	31	96	100	100	100	100
Human	65	0	0	12	37	81	88	91	100	100
Monkey	44	0	0	22	42	52	60	100	100	100
Rat	30	0	0	0	0	0	0	10	50	100
Rabbit	20	0	0	0	0	0	50	100	100	100

Serum (1 ml. of 1/10) incubated with carrageenin solution (1 ml.) for 30 minutes: EA (1 ml.) and buffer (4·5 ml.) added and incubation continued for a further 60 minutes. Both Ca^{++} and Mg^{++} added to buffer; red cells optimally sensitized for guinea-pig C′ activity.

* Concentration of carrageenin (μg./ml.).

Higher titres with rat C′ can be obtained if calcium is omitted from the buffer and a higher concentration of haemolysin used to sensitize the red blood cells (Osler *et al.*, 1957). We have confirmed this observation. The mean titre of thirty-one specimens of rat C′ estimated under these conditions was 84±2·9 $C'H_{50}$/ml. whereas under conditions suitable for the guinea-pig twenty-four samples gave titres of only 32±2·0 $C'H_{50}$/ml. When the

inhibitory activity of carrageenin was tested against rat C′ under these conditions of a higher level of sensitization and with added magnesium but no added calcium, a bi-phasic effect was observed, small amounts of carrageenin inhibited haemolysis whereas larger amounts permitted full haemolysis (Table 7). The reason for this anomaly is being investigated.

TABLE 7

INHIBITION OF RAT COMPLEMENT BY CARRAGEENIN

Concentration of carrageenin (μg./ml.)	*Haemolysis (%)*
400	100
200	93
100	68
50	27
25	21
12·5	21
6·25	34
3·125	77
1·56	93
0·78	100

Serum (1 ml. of 1/10) incubated with carrageenin solution (1 ml.) for 30 minutes: (EA 1 ml.) and buffer (4·5 ml.) added and incubation continued for a further 60 minutes. Mg^{++} but not Ca^{++} added to buffer: red cells optimally sensitized for rat C′ activity.

DISCUSSION

Lepow *et al.* (1963) isolated three fractions of the C'_1 complex of human complement. One of these fractions (C'_{1q}) appears to be identical with the 11S component isolated by Müller-Eberhard and Kunkel (1961). C'_{1r} could not be identified with any known complement activity and was presented as a new component. C'_{1s} was identified with C'_1 pro-esterase. All three fractions were necessary for the generation of C'_1 esterase activity.

Carrageenin, which allows cells in the form EAC'_1 to lyse in the presence of R1 ($C'_{4,2}$ and C'_3) has now been shown to prevent the formation of EAC'_1 by guinea-pig complement. This it could do either by inactivating directly some part of guinea-pig C'_1 or by preventing the sensitized cell from fixing C'_1 by an action on the cell itself. It is difficult at present to separate these two possibilities but the progressive inactivation of C′, illustrated in Fig. 1, suggests that carrageenin acts directly on complement rather than on the cells. Sub-fractionation of C'_1 has been achieved so far only with human complement. If guinea-pig C'_1 has an analogous composition then carrageenin does not prevent the fixation of the component analogous to C'_{1q}, if this fraction, as in human serum, is fixed to the sensitized cell in the absence of calcium (Müller-Eberhard, 1961). The effect of carrageenin on guinea-pig fractions analogous to C'_{1r} and C'_{1s} can only be determined when these fractions become available.

The antagonism of the anti-complementary effect of carrageenin by protamine is probably the result of an acidic molecule (carrageenin) reacting with a basic molecule (protamine) but it is tempting to speculate that the component of complement which is inhibited by carrageenin may also be a basic molecule.

inhibitory activity of carrageenin was tested against rat C′ under these conditions of a higher level of sensitization and with added magnesium but no added calcium, a bi-phasic effect was observed, small amounts of carrageenin inhibited haemolysis whereas larger amounts permitted full haemolysis (Table 7). The reason for this anomaly is being investigated.

TABLE 7

INHIBITION OF RAT COMPLEMENT BY CARRAGEENIN

Concentration of carrageenin (μg./*ml.*)	*Haemolysis* (%)
400	100
200	93
100	68
50	27
25	21
12·5	21
6·25	34
3·125	77
1·56	93
0·78	100

Serum (1 ml. of 1/10) incubated with carrageenin solution (1 ml.) for 30 minutes: (EA 1 ml.) and buffer (4·5 ml.) added and incubation continued for a further 60 minutes. Mg^{++} but not Ca^{++} added to buffer: red cells optimally sensitized for rat C′ activity.

DISCUSSION

Lepow *et al.* (1963) isolated three fractions of the C'_1 complex of human complement. One of these fractions (C'_{1q}) appears to be identical with the 11S component isolated by Müller-Eberhard and Kunkel (1961). C'_{1r} could not be identified with any known complement activity and was presented as a new component. C'_{1s} was identified with C'_1 pro-esterase. All three fractions were necessary for the generation of C'_1 esterase activity.

Carrageenin, which allows cells in the form EAC'_1 to lyse in the presence of R1 ($C'_{4,2}$ and C'_3) has now been shown to prevent the formation of EAC'_1 by guinea-pig complement. This it could do either by inactivating directly some part of guinea-pig C'_1 or by preventing the sensitized cell from fixing C'_1 by an action on the cell itself. It is difficult at present to separate these two possibilities but the progressive inactivation of C′, illustrated in Fig. 1, suggests that carrageenin acts directly on complement rather than on the cells. Sub-fractionation of C'_1 has been achieved so far only with human complement. If guinea-pig C'_1 has an analogous composition then carrageenin does not prevent the fixation of the component analogous to C'_{1q}, if this fraction, as in human serum, is fixed to the sensitized cell in the absence of calcium (Müller-Eberhard, 1961). The effect of carrageenin on guinea-pig fractions analogous to C'_{1r} and C'_{1s} can only be determined when these fractions become available.

The antagonism of the anti-complementary effect of carrageenin by protamine is probably the result of an acidic molecule (carrageenin) reacting with a basic molecule (protamine) but it is tempting to speculate that the component of complement which is inhibited by carrageenin may also be a basic molecule.

The lack of effect of *in vivo* inhibition of complement by carrageenin on anaphylactic shock could have three possible explanations.

(a) The small residual C′ activity (less than 2 $C'H_{50}$/ml.) could still be sufficient to permit the subsequent stages to progress.

(b) The component of complement inhibited by carrageenin, although essential for haemolysis, is not required for the anaphylactic reaction, or

(c) C′ is not involved in anaphylaxis.

Similar arguments apply to the lack of effect of carrageenin on the formation of the permeability factor PF P and further experiments on this point are in progress. The very complex problem of the role of complement in allergic reactions has been reviewed by Osler (1961) and it is apparent that a solution to this problem has not yet been reached.

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Faecal excretion of degraded and native carrageenan by the young rat

Hawkins & Yaphe (1965) have shown that when native, undegraded carrageenan is fed to young rats, at levels of 2 to 20% in the diet, it is 90–100% excreted in the faeces. No comparable results appear to be available for degraded carrageenan in the 16 000–19 000 number-average molecular weight range, and this report deals with the faecal excretion of both degraded and native iota-carrageenan by the young rat at a concentration of 5% in the diet. We have recently shown (Beattie, Blakemore & others, 1970) that when adult baboons were given a single large dose of 240 mg/kg of degraded carrageenan, which is more than three times the human dose in peptic ulcer treatment, no urinary metachromasia could be detected.

Carrageenans. The degraded carrageenan (code no. C16/L1927) was prepared from *Eucheuma spinosum* seaweed by Laboratoires Glaxo, Paris, and the native iota-carrageenan was a commercial product from Société Auby, Neuilly-sur-Seine, France. The analyses on the anhydrous basis are given in Table 1. The number-average molecular weight of C16 was in the 16 000 to 19 000 range (Blakemore & Dewar, 1970) and the weight-average molecular weight (by light scattering) was between 20 000 and 30 000 (Anderson & Soman, 1966). The molecular weight of the native carrageenan was unknown, but the inherent viscosity (η_{inh} in 0·1M NaCl solution) was about 18 times that of the degraded material.

Table 1. *Analyses of carrageenans*

Carrageenan	Inherent viscosity (η inh) (dl/g)	$[\alpha]_D$ in water	Total sulphate (SO_3Na), %	Sulphated ash, %	3,6-Anhydrogalactose ($C_6H_8O_4$), %
C16 degraded	0·41	+46·8°	37·9	29·7	18·1
Native	7·43	40	34·8	30·4	21·9

Animal experiments. Fifteen young male albino rats (Charles River C.D. strain) about 115 g, were randomly allocated to three treatment groups: 5% native carrageenan in the diet, 5% degraded carrageenan in the diet, and control diet without carrageenan. Spillers Laboratory Small Animal Diet (SG1/41B) in powdered form was mixed with the powdered degraded or native carrageenan, and 10 g balls were prepared by mixing with a known quantity of water and then drying at less than 60° to remove the water.

The animals were caged singly in suspended metal cages with a wire mesh floor through which scattered food and excreta could fall on to absorbent material beneath. Scatter of food or of faecal pellets between cages was prevented by placing folded sheets of blotting paper beneath each cage. Two balls (20 g) of each diet were placed in the food hopper daily as the sole source of food and the animals had water freely. After each 24 h, food consumption was measured. The feeding experiment lasted ten days, and on the tenth day, the 24 h faecal samples from each animal were collected, dried in a vacuum over phosphoric oxide to constant weight, and analysed for 3,6-anhydrogalactose (Black, Blakemore & others, 1965). The carrageenan content was calculated from this result after correcting for a mean "3,6-anhydrogalactose" value for the control faeces. The validity of the method was confirmed for 2 animals by estimation of total ester sulphate (Black & others, 1965).

The food intakes (Table 2) for the first seven and last three days and the weight gains for each animal in the three groups, indicated that there were no meaningful differences between animals receiving native and degraded carrageenan, although the mean weight

gain of the control group was significantly higher than that of the test groups. The mean daily consumption of each rat over the last three days was taken as the mean food intake over the 24 h period of faecal collection. Again (Table 3), there were no significant differences in any parameters between animals receiving native carrageenan and those receiving degraded carrageenan. The presence of carrageenan in the diet was readily detected by the increased weight of faeces compared to that of the control group. The faecal excretions of both native and degraded carrageenans were less than 100%, and the low figures may be partly attributed to small errors in measuring the

Table 2. *Food intake and weight gain in groups of young rats fed native and degraded carrageenan in the diet*

Animal no.	Treatment group	Initial wt (g)	Final wt (g)	Over 10 day period on test: Weight gain (g)	Food intake (g/day) over 7 days	Food intake (g/day) over 3 days
1	5% Native carrageenan	118	177	59	17·7	18·7
3		105	167	62	16·0	16·9
6		110	178	68	16·3	19·3
13		107	157	50	14·5	14·9
15		117	180	63	16·9	19·5
Mean		111·4	171·8	60·4	16·3	17·9
± s.e.*		2·6	4·3	3·0	0·5	0·9
4	5% Degraded carrageenan	106	175	69	18·2	18·9
7		114	174	60	18·3	19·1
10		118	185	67	18·0	19·1
12		120	192	72	19·0	19·8
14		126	162	36	18·6	19·4
Mean		116·8	177·6	60·8	18·4	19·3
± s.e.*		3·3	5·1	6·5	0·2	0·2
2	Control	112	196	84	16·9	19·3
5		112	190	78	15·5	16·8
8		127	196	69	16·5	17·2
9		117	198	81	15·6	19·9
11		120	201	81	16·3	17·2
Mean		117·6	196·2	78·6	16·2	18·1
± s.e.*		2·3	1·8	2·6	0·3	0·6

* s.e. = standard error

Table 3. *Analyses of faeces and excretion of carrageenans over 24 h.*

Animal no.	Treatment group	Mean food intake (g 24 h)	Dry faeces (g)	Faeces, % of food intake	Carrageenan in faeces %	Carrageenan excretion %	Excretion range %	Mean excretion %
1	5% native carrageenan in diet	18·7	4·900	26·2	16·7	88	68–88	80 ±3·5 (s.e.)
3		16·8	4·631	27·6	15·5	85		
6		19·3	5·366	27·8	13·9	77		
13		14·9	4·545	29·5	13·4	82		
15		19·5	4·785	24·5	13·9	68		
4	5% degraded carrageenan in diet	18·9	4·375	23·1	17·8	82	82–95	87 ±2·4 (s.e.)
7		19·0	4·513	23·8	18·4	88		
10		19·1	5·054	26·4	17·9	95		
12		19·8	4·368	22·2	18·4	82		
14		19·4	4·362	22·5	19·4	87		
2	Control: rat cake only in diet	19·3	3·145	16·3				
5		16·8	2·672	15·9				
8		17·2	2·420	14·1				
9		19·9	4·183	21·0				
11		17·2	2·815	16·3				

daily food intake and to assessing accurately the "3,6-anhydrogalactose" figure for the control faeces blank (0·37% as $C_6H_8O_4$).

The above results show that, when native and partially degraded iota-carrageenans were fed to young rats, at a concentration of 5% in the diet, there were no significant differences in the faecal excretion of the two polymers and the weight gained by the animals.

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Effects of Gum Guar, Locust Bean Gum and Carrageenan on Liver Cholesterol of Cholesterol-Fed Rats.* (27585)

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Our previous studies(1) indicate that pectin N.F. when fed at a 5% level in the diet largely counteracted the increment in liver cholesterol and liver total lipids induced by cholesterol feeding in the rat. Other roughage or bulk-forming materials such as cellulose, agar, sodium alginate, protopectin and calcium silicate (Micro-Cel) were ineffective in this regard. Data are presented here indicating that Gum Guar, Locust Bean Gum and carrageenan also have significant activity in counteracting the increment in liver cholesterol and liver total lipids induced by cholesterol feeding in the rat.

Procedure. The basal ration consisted of sucrose, 61%; casein,† 24%; cottonseed oil, 10%; salt mixture,‡ 5%; and the following vitamins per kg of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; 2-methyl-1,4 naphthoquinone, 5 mg; vit B_{12}, 150 μg; choline chloride, 2 g; vit A, 5000 U.S.P. units; vit D_2, 500 U.S.P. units; and alpha-tocopheryl acetate, 100 mg. Tests were conducted with rats fed the basal ration, the basal ration + 1% cholesterol, and the basal ration + 1% cholesterol + the various supplements indicated in Table I. The cholesterol and test supplements were incorporated in the basal ration in place of an equal amount of sucrose. Fifty-six male rats of the Holtzman strain with an average body wt of 43.6 g (range 38 to 50 g) were divided into 7 comparable groups of 8 each, placed in metal cages with raised screen bottoms (2 or 3 rats per cage), and provided the test diets and water *ad libitum.* Animals were fed on alternate days and all food not consumed 48 hours after feeding was discarded. Body weights were recorded weekly. After 28 days of feeding, the rats were anesthetized with sodium pentobarbital, and blood was withdrawn from the aorta into a heparinized syringe. Livers were excised, blotted to remove excess blood, weighed and stored in a freezer until analyzed. Lipid was extracted from the livers by the method of Thompson *et al.*(2), and cholesterol was determined on liver and plasma by the method of Nieft and Deuel(3). Total lipids were determined gravimetrically on an aliquot of the liver extract.

Results. The increment in liver cholesterol and liver total lipid induced by cholesterol feeding in the rat was largely counteracted by the concurrent feeding of Gum Guar, Locust Bean Gum or carrageenan at a 10% level in the diet. The effects of these supplements were similar to, although slightly less marked than, that of a comparable amount of pectin N.F. Cholesterol feeding also induced a slight increment in plasma cholesterol levels which was partially counteracted by concurrent feeding of pectin N.F. and several of the other test supplements. Differences between the various groups however were not statis-

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† Vitamin-Free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Hubbell, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE I. Effects of Dietary Supplements on Plasma and Liver Cholesterol and Liver Total Lipid of Cholesterol-Fed Rats (8 Animals/Group).*

Supplements fed with basal ration	Body wt at sacrifice, g†	Plasma cholesterol, mg/100 ml†		Liver cholesterol, mg/g†		Liver total lipid, %†
		Free	Total	Free	Total	
None	197.4 ± 4.9	20.1 ± 1.6	85.2 ± 5.8	1.63 ± .09	2.09 ± .09	4.74 ± .3
1% cholesterol	201.3 ± 6.8	21.0 ± 1.1	105.5 ± 5.0	2.72 ± .14	16.17 ± 1.42	9.03 ± .7
Idem + following supplements:						
5% pectin N.F.	185.4 ± 6.1	19.6 ± 1.0	92.8 ± 4.2	2.45 ± .13	5.59 ± .64	6.31 ± .4
10% " "	181.7 ± 5.6	18.2 ± 1.4	95.6 ± 3.3	2.38 ± .17	4.12 ± .42	4.78 ± .3
10% Gum Guar	177.6 ± 5.2	20.8 ± 1.2	91.3 ± 4.3	2.29 ± .12	6.05 ± .79	6.29 ± .5
10% Locust Bean Gum	180.3 ± 5.4	24.0 ± 1.5	103.3 ± 5.0	2.38 ± .08	7.59 ± .73	6.81 ± .4
10% carrageenan	191.9 ± 3.8	20.8 ± 1.6	89.3 ± 5.5	2.22 ± .09	5.52 ± .62	5.86 ± .3

* Test supplements obtained from the following sources: pectin N.F. (citrus), Sunkist Growers, Ontario, Calif.; Gum Guar and Locust Bean Gum, Hathaway Allied Products, Los Angeles, Calif.; carrageenan (Gelcarin MR 100), Marine Collids, Inc., of America, New York. Test supplements were all natural sources of hydrophyllic complex carbohydrate colloids made up of repeating units of (1) galacturonic acid in the case of pectin, (2) mannose and galactose in Gum Guar and Locust Bean Gum, and (3) sulphated galactose in Gelcarin MR 100. The pectin N.F. employed was a purified material obtained from the dilute acid extract of the inner portion of the rind of citrus fruits. It had a methoxyl value of 10.7% on a moisture-ash-free basis. Gum Guar was the ground endosperm of Guar (known botanically as *Cyamopsis tetragonolaba*) seed with a galactomannan content of approximately 80%. Locust Bean Gum was obtained from the endosperm of the kernels of the Carob tree. It was a hemicellulose product of about 4 parts mannose and 1 part galactose. Gelcarin MR 100, a highly purified product designed for use in milk or milk products, is known chemically as carrageenan. The latter occurs naturally in a number of red seaweeds (class Rhodophyceae) but is obtained principally from the group of seaweeds known as Irish moss.

† Including stand. error of mean.

tically significant.§ Findings are summarized in Table I.

Discussion. A number of studies indicate that, in general, populations habitually subsisting on diets low in fats and animal protein tend to have a low concentration of serum cholesterol and a low incidence of cardiovascular disease(4-7). Such diets contain a number of constituents which are either absent from or present in only minute amounts in the diets of populations with a high incidence of hypercholesterolemia, atherosclerosis and coronary artery disease. Walker and Arvidsson(8) and Higginson and Pepler(9) were among the first to call attention to the high fiber content of the Bantu diet as a possible explanation for the low serum cholesterol level observed in the Bantu population. A similar suggestion was also made by Bersohn *et al.*(10). More recently Keys *et al.* (11) conducted controlled experiments in which groups of physically healthy men subsisted alternately on "American" and "Italian" types of diets, devised to be comparable in calories, proteins and in kind and amounts of fat but differing in the sources of carbohydrates. An abundance of fruits and vegetables in the Italian type diets (which tended to be high in complex carbohydrates such as pectins, hemicelluloses and fiber) replaced equivalent calories in simpler carbohydrates in the American type. Serum cholesterol lev-

§ Food intake was determined for rats in the various groups. Since the weight increment of rats fed the test supplements was less than that of rats fed the basal + cholesterol diet, the question arose whether the reduction in weight increment of rats fed the test supplements may not have been due to a reduction in amount of diet and hence cholesterol ingested and whether the reduced cholesterol intake in turn may not have been the cause of lower liver cholesterol and liver total lipid levels. Such, however, does not appear to be the case, for differences in body weight between the various groups were not statistically significant whereas differences in liver cholesterol and liver total lipid were. Furthermore, an even greater reduction in body weight was observed (unpublished findings) in rats fed the basal ration + cholesterol + 20% alfalfa meal without an accompanying reduction in liver cholesterol and liver total lipid values. The possibility that the test supplements inhibited absorption of cholesterol from the gut, however, has not been excluded.

els were significantly lower with the "Italian" type diets. In subsequent studies Keys *et al.* (12) reported that citrus pectin when fed at a level of 15 g per day caused a slight but statistically significant reduction in serum cholesterol levels in physically healthy, middle-aged men; cellulose (fiber) fed under comparable conditions was without significant effect. These studies suggest that cellulose or fiber *per se* was not responsible for the low serum cholesterol levels of the Bantu and comparable groups, but that pectin, another complex bulk-forming carbohydrate, may have had some activity in this regard.‖ An analysis of the diets of native populations with low serum cholesterol levels indicated that in addition to pectin such rations also contain gums and/or other complex carbohydrates such as colloids of marine plants which were found in the present experiment to cause a highly significant reduction in liver cholesterol levels in the cholesterol-fed rat. Further studies are indicated to determine what effect these substances might have, when administered alone or in combination with one another and pectin, in treatment of hypercholesterolemia and atherosclerosis in man.

‖ Unpublished studies from this laboratory indicate that different batches of pectin may vary markedly in anti-cholesterol activity. Pectic preparations with a methoxyl content of 5% or less were without activity in counteracting the increment in plasma and liver cholesterol levels induced by cholesterol feeding in the rat in contrast to the marked activity exhibited by pectin N.F. preparations of relatively high methoxyl content (10.7% on a moisture-ash-free basis).

Summary. The increment in liver cholesterol and liver total lipid induced by cholesterol feeding in the rat was largely counteracted by concurrent feeding of Gum Guar, Locust Bean Gum or carrageenan at a 10% level in the diet. Effects were similar to, although slightly less marked, than that obtained with a comparable amount of pectin N.F.

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Identification of Stabilizing Agents

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EXTENSIVE use of a variety of polysaccharides as stabilizing or thickening agents in foods has created a need for analytical methods for the identification of commercially available materials and for their detection and quantitative estimation in food products. A number of stabilizers, which are used or have been suggested for use in foods, are listed and their chemical nature is indicated in Table I. Throughout this report the term "gums" is used in referring to all of these materials. Gelatin is included because it has many uses similar to those of the polysaccharides.

As the initial step in a project concerned with the development of the required analytical procedures, methods for the qualitative identification of a number of gums have been studied. Several procedures for the identification of gums are found in the literature. A method described by Jacobs and Jaffe (*15*) classified the gums on the basis of physical characteristics or appearance of precipitates and thus requires considerable experience on the part of the analyst. Their outline does not include pectic substances, alginates, methylcellulose, carboxymethylcellulose, gum ghatti, starch, or gelatin. It also has the disadvantage of using an unstable reagent (Millon's) which must be freshly prepared each day. A method developed by Cannon and adopted

The present work was undertaken to develop a method for the identification of stabilizing and thickening agents used in food products. The materials studied were pectin, de-esterified pectin, algin, Irish moss, gum tragacanth, gum karaya, locust bean gum, starch, agar, gum arabic, gum ghatti, carboxymethylcellulose, methylcellulose, and gelatin. A proposed identification scheme is based on precipitation reactions with calcium chloride, sodium hydroxide, barium hydroxide, and lead acetate. In addition, reactions of the stabilizing agents with a cationic soap, ammonium sulfate, mercuric nitrate, papain, and gelatin are listed. The proposed scheme should be useful for identification of unknown stabilizing agents. A number of the reactions reported might be employed for the identification of individual stabilizing agents in mixtures of these materials or isolated from foods.

by the Association of Official Agricultural Chemists (*3, 5*) does not provide for the identification of pectic substances, alginates, methylcellulose, carboxymethylcellulose, locust bean (carob) gum, or gum ghatti. Bryant (*4*) has described a procedure for distinguishing between pectin and certain gums, but it does not provide for positive identification of the gums. A number of other publications dealing with characteristic properties of these polysaccharides have been summarized by Mantell (*16*), but a systematic procedure is still needed for their identification. Such a procedure would be useful for the identification of products used as thickeners or stabilizers in foods, drugs, and cosmetics and ultimately for the identification of polysaccharides isolated from these materials.

In the present investigation, the manner in which the gums disperse in water after being wetted with alcohol has been a valuable index to the identity of unknown samples. Their solubility properties are summarized in Table II. Use has also been made of the fact that many of the polysaccharides occur as salts of complex organic acids (Table I). The acidic properties may be due to the presence of uronic acid groups, as in gum arabic, or to the unesterified portion of sulfuric acid molecules esterified with the polysaccharide. When mineral acids are added to aqueous solutions or dispersions of these salts, the effective concentrations of the polysaccharide anions are decreased. Thus, although the complex anions may yield insoluble salts with heavy metal cations, most are not precipitated from acid solutions. The amount the pH must be raised in order to precipitate the heavy metal salts—e.g., barium, mercury, or lead—is frequently characteristic of the individual polysaccharide.

Table I. Source and Chemical Nature of Materials Commonly Used as Thickening Agents in Foods

Material	Source	Principal Components	References
Pectic substances	Fruits	Galacturonic acid (occurs as methyl ester)	(*18*)
Algin (sodium alginate)	Seaweeds	Mannuronic acid (Na salt)	(*16*)
Irish moss	Seaweeds	Galactose, galactose 4-sulfate (K and Ca salts)	(*18*)
Agar	Seaweeds	Galactose (D- and L-), galactose 6-sulfate (Ca and Mg salts)	(*18*)
Tragacanth	Plant gum	L-Fucose, D-xylose, galacturonic acid, L-arabinose, D-galactose	(*18*)
Methylcellulose	Modified cellulose	Methyl-D-glucose	(*18*)
Starch	Plants	D-Glucose	(*18*)
Carboxymethylcellulose	Modified cellulose	Carboxymethyl-D-glucose	(*18*)
Locust bean gum (carob gum)	Seed endosperm	Mannose and galactose	(*16*)
Guar gum	Seed endosperm	Mannose and galactose	(*20*)
Karaya	Plant gum	Galactose, acetic acid, galacturonic acid, rhamnose, tagatose	(*14*)
Arabic (acacia)	Plant gum	D-Glucuronic acid, D-galactose, L-arabinose, rhamnose (mixed Ca, Mg, and K salts)	(*18*)
Ghatti	Plant gum	L-Arabinose, galactose, galacturonic acid (Ca salt)	(*16*)
Gelatin	Modified protein	Amino acids	

EXPERIMENTAL

During the present investigation 0.5 to 1.0% aqueous dispersions of the polysaccharides were used for the tests. Aliquots of from 3 to 5 ml. were treated with varying concentrations of the reagents which it was hoped would give characteristic precipitation reactions. Initially the reagents used were those for which Jacobs and Jaffe (*15*) have described reactions with several polysaccharides. Subsequently a number of other reagents were used.

Table II. Dispersion in Water of Gums, Wetted with Alcohol

Gum	Manner of Dispersal in Water
Pectic acid	Insoluble
Pectate (Na, K, or NH_4 salts)	Forms either clear or turbid solution on heating
Pectate (Ca salts)	Insoluble
Pectin	Swells in cold water and dissolves on heating
Alginate	Dissolves slowly in cold water or quickly on heating to form viscous solution
Irish moss	Dissolves slowly in cold water, rapidly on heating to form viscous solution
Agar	Swells in cold water, dissolves on heating, gels on cooling
Tragacanth	Swells to form viscous dispersion in cold or hot water, but does not form true solution
Methylcellulose	Dissolves slowly in cold water but becomes cloudy or gels on heating
Starch	Disperses on heating
Carboxymethylcellulose	Dissolves slowly in cold water, rapidly on heating, giving clear viscous solution with some fine fibrous suspended material
Locust (carob)	Forms viscous suspension but not a true solution
Karaya	Forms viscous suspension. Insoluble particles settle on standing
Arabic (acacia)	Dissolves in cold water to form a clear only slightly viscous solution
Ghatti	Dissolves to form almost clear solution but some insoluble material may remain as fine suspension
Gelatin	Swells in cold water and dissolves on heating

Reactions which were found useful for characterizing the gums are summarized in Tables III and IV. Only those materials having anionic components, such as alginates, or potential anionic components, such as pectin, give pronounced reactions with cationic soap (Table III). As in the case of precipitates with heavy metals, the precipitates with the cationic soap quickly disperse on acidification of the medium. Ammonium sulfate is of interest, in that it gives pronounced precipitation tests with several of the gums but not with alginates, pectin, tragacanth, karaya, arabic, or ghatti, each of which probably contains uronic acid components. The reactions with Stokes's acid mercuric nitrate illustrate the effects of low pH on precipitation of heavy metal salts of the polysaccharide acids. An excess of the reagent makes the solutions strongly acidic and thus the weakly dissociated acids redisperse. Alginic acid and pectic acid are insoluble and thus are not dissolved by excess Stokes reagent. Papain and gelatin give pronounced precipitation reactions only with those gums having anionic components. These precipitates are found only if the

Table III. Precipitation Reactions of Polysaccharide Gums and Gelatin

Gum	1 Vol. 1% Solution of Cationic Soap[a]	0.5 Vol. Saturated Ammonium Sulfate	Diluted[b] Stokes's Acid Mercuric Nitrate Added Dropwise	1 Vol. 2% Papain (6)[c]	1 Vol. 2% Gelatin[c]	4 Vol. 95% C_2H_5OH + 2 – 3 Drops Saturated NaCl
De-esterified pectin	Fine opaque precipitate	Gelatinous translucent precipitate	Gels (almost opaque). Insoluble in excess reagent	Precipitate	Precipitate	Gelatinous precipitate, gels (1 vol.)
Alginate	Fine opaque precipitate	Nil	Gels (almost opaque). Insoluble in excess reagent	Precipitate	Precipitate	Gelatinous precipitate (1 vol.) becomes stringy with 4 vol. alcohol
Pectin	Flocculent precipitate	Nil	Forms almost opaque gel which dissolves in excess reagent	Cloudy	No definite effect	Transparent gelatinous precipitate. Gels (1 vol.)
Irish moss	Stringy or flocculent precipitate	Gelatinous precipitate or gel	Transparent gel. Redispersed by excess reagent	Precipitate	Precipitate	Stringy precipitate
Agar	Gelatinous precipitate	Flocculent precipitate	Turbid or cloudy	Cloudy	Precipitate	Fine flocculent precipitate
Tragacanth	Fine opaque precipitate	Nil	Flocculent precipitate. Dissolves in excess reagent	Precipitate	Precipitate	Voluminous precipitate, jellylike
Methylcellulose	Nil	Precipitate	Nil	Nil	Nil	Nil
Starch	Nil	Precipitate	Nil	Nil	Nil	Opaque flocculent precipitate
Carboxymethylcellulose	Gelatinous clotted precipitate	Gelatinous precipitate	Precipitate dissolves in excess reagent	Precipitate	Precipitate	Voluminous clotted precipitate
Locust	Nil	Precipitate (voluminous)	No pronounced effect	No pronounced effect	Nil	Voluminous opaque stringy precipitate, forms clot
Karaya	Flocculent precipitate	Nil	Flocculent precipitate dissolves in excess reagent	Precipitate	Precipitate	Flocculent precipitate, discrete particles
Arabic (acacia)	Precipitate (very fine)	Nil	Flocculent precipitate dissolves in excess reagent	Precipitate	Precipitate	Fine opaque nonsettling precipitate
Ghatti	Fine precipitate	Nil	Nil	Nil	Precipitate	Fine precipitate, nonsettling (2–3 vol.)
Gelatin	Precipitate in alkaline medium (22)	Precipitate	Nil	Nil	Nil	Finely flocculent precipitate, coagulates

[a] Rodalon (alkyl dimethyl benzyl ammonium chloride), Fairfield Laboratories, Plainfield, N. J.
[b] Mercury dissolved in twice its weight of concentrated nitric acid and diluted to 100 times its volume with distilled water.
[c] Precipitates with papain and gelatin are observed only in weakly acidic medium and most exhibit properties of coacervates rather than true precipitates.

pH of the mixture is below the isoelectric point of the protein and it is possible that they would be more correctly called coacervates. They are usually dispersed by a few drops of mineral acid or of dilute ammonium hydroxide. The characteristic manner in which some of the gums are precipitated by alcohol may also be of value in their identification.

The reactions described in Table IV form the basis of a proposed procedure for the systematic identification of the gums.

REAGENTS

Calcium chloride ($CaCl_2$), 3% solution (weight/volume).
Ammonium hydroxide, 3.0 *N* solution.
Sodium hydroxide, 3.0 *N* solution.
Barium hydroxide, saturated solution stored in a bottle equipped with a siphon and a soda-lime tube.
Basic lead acetate, 20% suspension (weight/volume). Heat to boiling, cool, and use supernatant solution.
Hydrochloric acid (or other mineral acid), 3.0 *N* solution.
Methylene blue, 0.1% aqueous solution.
Tincture of iodine (U.S.P., *14*).
Iodine–potassium iodide stock solution, containing 0.5% iodine and 1.0% potassium iodide. Iodine–potassium iodide test solution, consisting of stock solution diluted 1 to 5.
Cupric sulfate ($CuSO_4.5H_2O$), 15% solution (weight/volume).
Borax ($Na_2B_4O_7.10H_2O$), 4% solution.
Ruthenium red test solution (*3*).
Picric acid, saturated aqueous solution.

IDENTIFICATION PROCEDURE

Wet a 0.25- to 0.5-gram sample of the material to be identified with 1 to 2 ml. of 95% alcohol and add 50 ml. of distilled water. Suspend the solid material in the water by shaking or stirring. Heat the suspension, with frequent shaking, on a hot plate or over a burner. If the sample dissolves, discontinue heating immediately; otherwise hold at 85° to 95° C. for 15 minutes.

Group A. I. Treat a 3- to 5-ml. aliquot of the solution with 0.2 volume of 0.25 *M* calcium chloride. A gelatinous precipitate or gel indicates alginates or de-esterified pectin.

If no reaction is apparent with calcium chloride alone, add 1 volume of 3 *N* ammonium hydroxide to the calcium chloride treated sample. Slow formation of a gel or gelatinous precipitate indicates pectin.

II. If either test in A I was positive, mix a fresh 3- to 5-ml. aliquot of sample with 1 volume of 3.0 *N* sodium hydroxide. Observe the reaction and then heat the mixture in a boiling water bath for 10 minutes.

Immediate formation, in the cold, of a gelatinous or flocculent precipitate indicates either pectin or de-esterified pectin. No precipitate indicates alginates. All three mixtures become yellow on heating, but the precipitates with pectic substances do not dissolve.

Group B. If the material is not an alginate or a pectic substance, carry out the following tests.

I. Mix a 3- to 5- ml. aliquot of the sample with 0.1 volume of saturated barium hydroxide. Observe in the cold and heat in boiling water bath for 10 minutes.

Formation in the cold of a nonsettling, almost opaque, gelatinous precipitate indicates Irish moss. Carry out confirmatory test for Irish moss.

A small amount of flocculent precipitate or cloudiness in the cold and a definite lemon yellow color on heating identify gum tragacanth.

Color changing during heating to yellow, then to green, and finally to gray indicates agar. Carry out confirmatory test for agar.

If the mixture becomes cloudy or forms a gel on heating, but becomes clear on cooling, methylcellulose is indicated. Carry out confirmatory test for methylcellulose.

An opaque flocculent precipitate which may tend to redisperse on heating and reprecipitate on cooling indicates starch. Carry out confirmatory test for starch.

Precipitates which disappear when the barium hydroxide is thoroughly mixed with the sample may be disregarded at this point.

II. If the material has not been identified, mix a fresh 3- to 5-ml. aliquot of sample with 1 volume of saturated barium hydroxide. Observe whether there is an immediate precipitation and examine again after standing 5 minutes at room temperature.

Table IV. Precipitation Reactions Used as Basis of Proposed Procedure for Identification of Gums

(Reactions used as identification tests within heavy lines)

Gum	1/2 Vol. 2.5% $CaCl_2$	1/2 Vol. 2.5% $CaCl_2$ + 1/2 Vol. 3 *N* NH_4OH	1 Vol. 3 *N* NaOH	1/10 Vol. Saturated $Ba(OH)_2$ Cold	1/10 Vol. Saturated $Ba(OH)_2$ Heated	1 Vol. Saturated $Ba(OH)_2$	1/2 Vol. Basic Lead Acetate	Basic Lead Acetate + 1/2 Vol. 3*N* NH_4OH	Confirmatory Tests
De-esterified pectin	Gelatinous ppt. or gel		Gelatinous or flocculent ppt., yellow color on heating	Gel or gelatinous ppt.	Ppt. and soln. turn yellow	As for 1/10 vol.	Gels	As for lead acetate alone	Gelatinous ppt. when acidified with mineral acids
Alginate	Gelatinous ppt. or gel		Clear yellow soln. on heating	Gel or gelatinous ppt.	Ppt. and soln. turn yellow	As for 1/10 vol.	Gels	As for lead acetate alone	Gelatinous ppt. when acidified with mineral acids
Pectin		Gelatinous ppt. forms slowly	Gelatinous or flocculent ppt., yellow color on heating	Gel or gelatinous ppt.	Ppt. and soln. turn yellow	As for 1/10 vol.	Translucent gel	As for lead acetate alone	
Irish moss	Some samples may give flocculent ppt.			Gelatinous ppt. or gel, almost opaque	Ppt. may flocculate with prolonged heating	Gelatinous ppt. or gel	Gelatinous ppt. or gel	As for lead acetate alone	Forms blue fibrous ppt. with aqueous methylene blue
Tragacanth				May be small amount of ppt.	Lemon yellow color	Flocculent ppt.	Flocculent ppt.	As for lead acetate alone	
Agar				Nil	Becomes yellow then green and gray	Nil	Flocculent ppt.	Gels	Gives blue or black stain with tincture of iodine
Methylcellulose				Nil	Becomes turbid or forms gel; becomes clear on cooling	Nil	Nil	Gels	Aqueous dispersions are not precipitated by alcohol, but form gel or become cloudy when heated
Starch				Opaque flocculent ppt.	May redisperse	Flocculent ppt.	Flocculent ppt.	Very heavy flocculent or gelatinous ppt.	Blue stain with I_2-KI reagent
Carboxymethylcellulose				Ppt. dissolves on shaking	Nil	Flocculent ppt.	Gels	As for lead acetate alone	Forms clotted ppt. with $CuSO_4$
Locust[a]						Clotted opaque ppt.	Opaque gel	As for lead acetate alone	Gels with 1/4 vol. 4% borax
Karaya						Flocculent ppt., forms slowly	Flocculent ppt.	As for lead acetate alone	Swells and stains pink with ruthenium red test solution
Arabic (acacia)							Voluminous opaque ppt.	As for lead acetate alone	Readily soluble in water at room temperature
Ghatti							May be small amount of flocculent ppt.	Voluminous opaque ppt.	Fine ppt. with 4 vol. of alcohol. Arabic also gives fine ppt.
Gelatin									Gives fine yellow ppt. when added to saturated picric acid

[a] Locust bean gum and guar gum give identical reactions and cannot be distinguished on basis of these tests.

Table V. Samples Examined by Proposed Identification Procedure

Gum	Type	No. of Samples
Sodium pectate	Powdered	1
Pectic acid	Granular	1
Pectin	Powdered	2
Sodium alginate	Powdered	5
Irish moss	Powdered	2
Tragacanth	Shredded	1
Agar	Granular	1
	Fibrous	3
Methylcellulose		2
Starch	Whole wheat flour	1
	Tapioca flour	1
	Soluble starch	3
	Cornstarch	1
	Amioca (amylopectin)[a]	1
	Clear jel[a]	1
	Clear-Flo-II (sodium salt of starch acid ester)[a]	1
	Dry-Flo (starch ester)[a]	1
	Vulca (starch ether)[a]	1
	Melojel[a]	1
	Nu-film (starch acid ester)[a]	1
Sodium carboxymethylcellulose	Powdered	1
Locust bean (carob)	Powdered	7
Guar	Powdered	1
Karaya	Powdered	1
Arabic (acacia)	Powdered	3
	Lump	1
Ghatti	Powdered	1
	Lump	1
Gelatin	Granular	2

[a] Products of National Starch Products, New York, N. Y.

A voluminous opaque, stringy precipitate which tends to form a clot indicates locust bean gum. This precipitate may appear flocculent if the mixture is shaken vigorously. Carry out confirmatory test for locust bean gum.

A voluminous opaque flocculent precipitate which forms immediately indicates carboxymethylcellulose. Carry out confirmatory test for carboxymethylcellulose.

An opaque flocculent precipitate which forms slowly and is not voluminous indicates gum karaya. Carry out confirmatory test for karaya.

Group C. If the sample has not been identified it may be gum arabic, gum ghatti, or gelatin.

I. Mix a fresh 3- to 5-ml. aliquot of sample with 1 ml. of basic lead acetate solution. Immediate formation of a voluminous opaque precipitate indicates gum arabic.

If there was only a small amount of flocculent precipitate, or no precipitate, with the basic lead acetate add 1 ml. of 3.0 *N* ammonium hydroxide to the lead-containing sample. A voluminous opaque flocculent precipitate indicates gum ghatti. If there is no precipitate, the sample probably is gelatin. Carry out confirmatory test for gelatin.

CONFIRMATORY TESTS

Alginates and De-esterified Pectins. Add 0.2 volume of 3 *N* hydrochloric acid (or other mineral acid) to 3 to 5 ml. of the sample. A gelatinous precipitate confirms alginates or de-esterified pectin.

Irish Moss. Add 2 to 3 drops of 0.5% methylene blue in water to 1 ml. of the sample solution. Precipitation of purple-stained fibers confirms Irish moss.

Methylcellulose. Mix 5 ml. of sample with 25 ml. of 95% alcohol and 2 to 3 drops of saturated sodium chloride. No precipitate confirms methylcellulose.

Agar. Precipitate gum from 5 ml. of sample with alcohol and stain with tincture of iodine (*3*). Starch is also stained blue by this reagent.

Starch. Add 1 to 2 drops of the iodine potassium iodide test solution to 1 ml. of sample. A blue or purple color confirms starch. Some samples of gum tragacanth may give a faint blue test here.

Carboxymethylcellulose. Add 2 ml. of 1.0 *M* cupric sulfate to 3 to 5 ml. of sample solution. An opaque, slightly bluish, clotted precipitate confirms carboxymethylcellulose.

Locust Bean Gum. Add 1 ml. of 4% borax to 3 to 5 ml. of gum solution. If mixture gelatinizes, locust bean gum is confirmed. Guar gum also forms a gel here.

Karaya. Precipitate gum from 5 ml. of solution with alcohol and stain with ruthenium red (*3*). If sample swells considerably and is stained pink, karaya is confirmed.

Gelatin. Add 2 to 3 drops of gum solution to 2 ml. of saturated picric acid. A fine yellow precipitate confirms gelatin.

DISCUSSION

The proposed procedure has been tested with the gums listed in Table V. It was possible to identify the modified starches as starch products, because the solubility of all these materials is decreased by barium hydroxide and all give positive tests with the iodine-potassium iodide reagent. It is possible that not all the thickening agents or gums employed in food products at the present time have been included in this study. Cherry gum and quince seed gum, for example, have been suggested in the literature for use in foods. However, the proposed procedure includes all the gums that were available during the investigation.

The proposed scheme for identification of stabilizing and thickening agents is applicable only when they have not been mixed with other materials. To identify thickening agents in foods by this method it would first be necessary to separate them from the foods, but separation techniques might alter the reaction characteristics of the gums. Much work has been done on methods for the separation and detection of gums in particular foods, such as mayonnaise and French dressing (*2, 7, 10, 11*), soft curd cheese (*1, 8, 10, 11, 13, 19*), tomato products (*10, 13*), starchy foods (*21*), cacao products (*13, 17, 23*), ice cream and frozen desserts (*9–11*), canned chicken (*10*), and meat products (*12*). Additional references to methods for separating gums from foods may be found in reviews by Jacobs and Jaffe (*15*) and Mantell (*16*). The emphasis in most procedures has been on detection of the gums without identification. However, Wyler (*24*) has outlined methods for the detection and identification of locust (carob) bean gum, methylcellulose, carboxymethylcellulose, starch, pectin, and alginate in meat products. Thus identification tests proposed in the present paper may be useful for the identification of gums separated from foods by methods already described in the literature. However, it is probable that many special techniques will be required for the analyses of particular combinations of foods and thickeners. A great deal more work must be done before it will be possible to identify all of the gums in the various foods in which they may be used.

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COMMENTS ON THE MECHANISM OF THE FETOTOXIC EFFECT OF TRAGACANTH

(Über den Mechanismus der fetalschädigenden Wirkung von Traganth)

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A. Introduction:

Not all the substances, whose possible teratogenic effect must be tested, are soluble in water, physiological salt solution and Ringer's solution. Hence, they must be applied, in the form of suspensions or emulsions according to their mode of utilization, orally or parenterally. Natural substances, beside synthetic products like carboxymethyl cellulose, are used as suspensions in manufacturing these preparations. That is why the influence of suspensions involving natural substances was tested in comparison with distilled water and Ringer's solution as to gestation in mice. This was done especially since WILLIAMSON and Collaborators (1963), as well as CARTER (1965), proved in their tests conducted on chick embryos that inert substances, like sand, ground glass, colloidal Al or talc, produced malformations with the same frequency as definitely teratogenic substances.

B. Material and Methods:

1. Animal Species Used:

Mice of the NMRI-breed were used for the tests (animals from closed random breeding; Breeder Dr. Haakh, until late 1964: Federal Research Institution for Virus Diseases in Animals, since 1965: Firma Ivanovas, Kissleg, Allgäu). Upon reception, all the mice received a protective inoculation of active vaccine virus against ectromelia at the Industrial Hygiene Pharmacological Institute of the BASF (Zeller and Reckzeh, 1965).

2. Type of the Substances Tested and of the Substance Specimens:

Table 1 contains a survey of the substances tested and substance specimens, and reveals their origins.

Table 1: Survey of the substances tested

Bezeichnung	Chargen-nummer	Herkunft	
Aqua destillata	—		
Ringerlösung	—		
Gummi arabicum	G 1012	E. Merck, AG	Darmstadt
Agar	51581	E. Merck, AG	Darmstadt
Carrageen	—	F. Schulze u. Co	Mannheim
Guar	M 175	Meyhall Chemical AG	Kreuzlingen (Schweiz
Talkum	T 61754	E. Merck, AG	Darmstadt
persischer Traganth Nr. 0	—	E. Merck, AG	Darmstadt
persischer Traganth Nr. 1	434209	E. Merck, AG	Darmstadt
persischer Traganth Nr. 2	387011	E. Merck, AG	Darmstadt
persischer Traganth Nr. 3	263868	E. Merck, AG	Darmstadt
persischer Traganth Nr. 4	277520	E. Merck, AG	Darmstadt
persischer Traganth Nr. 5	419414	E. Merck, AG	Darmstadt
persischer Traganth Nr. 6	8021 65	Imhoff u. Stahl, GmbH	Mannheim
persischer Traganth Nr. 7	B 420 5165	Riedel de Haen, AG	Seelze-Hannover
indischer Traganth Nr. 1	14009/64	Imhoff u. Stahl, GmbH	Mannheim
indischer Traganth Nr. 2	562 65	Imhoff u. Stahl, GmbH	Mannheim

The terms "Persian tragacanth" designate the officinal tragacanths from the Near East. These are mucilages obtained mainly from Astragalus gummifer Labillardière and from related asiatic astragal species, all of them belonging to the Leguminosae family (TSCHIRSCH, 1912; BERGER, 1964). In previous time, this astragal mucus, called also "true tragacanth", was extended with "Indian tragacanth", and still today Indian tragacanth is often used as a substitute for the more expensive pure tragacanth.

Indian tragacanth, called also "false tragacanth", Karaya- or Sterculia-rubber, is obtained by scraping the bark of the trunk of Sterculia ureaus Roxb., a tree widely distributed in Hither India (Hindustan). Unlike true tragacanth, Sterculia-rubber contains no starch, possesses no adhesive power, and swells much more rapidly in water.

3. Nutrient Media:

Merck-Standard I-nutrient agar and Merck-Standard-nutrient broth were used in obtaining the bacteriological nutrient media. The various differential nutrient media were prepared according to the prescriptions indicated by HALLMANN ("Bakteriologische Nährböden" - Bacteriological Nutrient Media, Thieme-Verlag, Stuttgart, 1953).

4. Methode Used in Testing the Teratogenic Effect:

In teratogenic tests, the fertilization periods should be indicated as accurately as possible. Hence, in our Institute, when teratogenic tests are conducted on mice, several 100 pubescent female mice are placed together each time for 2 hours with the males (bucks) (5 females with one male), once a week from 10 to 12:00 AM. When the cohabitation is terminated, each animal exhibiting a vaginal plug is isolated. In the case of these "plug mices", the pairing day is called the 1st day of gestation (FROHBERG and OETTEL, 1964).

On the 19th day of gestation, thus one day before the normal littering day, all the treated or untreated mice are killed, and the number of the implantation places, of the intact and resorbed fetuses, as well as the number of the dead but externally uninjured fetuses, are determined. The implantation places, which can be recognized by the "metrial glands", which can be identified as quite perfused yellowish nodules on the mesometrium insertion of the uterine excrescences, and which are not occupied by living or artificially dead fetuses or fetal resorptions, characterize the number of the miscarried embryos.

The body length and weight of the fetuses are determined, then their malformations are examined macroscopically. This examination is conducted systematically from head to tail. Subsequently, the fetuses are fixed in alcohol; the soft parts are clarified in caustic potash and, in order to evaluate the individual bones, the skeletal system is dyed with alizarin red S by a modified Dawson method. These clarified and colored fetuses are kept in 100% glycerine DAB 6 (FROHBERG and OETTEL, 1966).

In order to be able to make a statement regarding the possible embryotoxic effect of a product in the case of the animal species used for the tests, one must possess an accurate knowledge of the spontaneous values. Hence investigations of the littering size, of the rates of fetal resorption and of malformation, were conducted on 413 pregnant and untreated NMRI mice.

In the uteri of 414 untreated animals, 4293 implantation places were found, that is 10.4 on an average per mother animal (See Table 2).

Table 2: Spontaneous values in 414 NMRI control-mice. The mother animals were caused to die on the 19th day of gestation.
a. Implantations b. Fetuses c. Alive d. Malformed e. Dead
f. Resorptions g. Miscarriages h. Total number i. Average per animal

	a. Implantationen	b. Feten lebend	c. mißgebildet	d. runts	e. tot	f. Resorptionen	g. Aborte
Gesamtzahl (h)	4293	3918	60	18	20	343	12
Durchschnitt pro Tier (i)	10,4	9,5	0,14	0,04	0,05	0,83	0,03

From the 4293 implanted embryos, 343 had died or had been resorbed. Thus the average quota of fetal resorption per animal was 0.83- One must bear in mind, however, that fetal resorptions were found only in 199 of the 414 control mice, and also one resorption in 120 animals, 2 resorptions in 44 mice, and more than 2 resorptions in 35 animals. One mouse, in each case, had even 6 or 8 spontaneous resorptions. On the 11th day of gestation, in a control animal, all the 12 fetuses were expelled as miscarriages.

For the most part, the mice had 9 - 13 fetuses per litter, and the 3918 living fetuses of the 414 control animals exhibited on an average a length of 2.2 cm and a body weight of 1.2 g. Eighteen living fetuses of the altogether 14 mother animals, must be regarded, with a body weight of only 0.6 - 0.8 g, as "congenital runts". In 18 mothers, altogether 20 dead, nonmalformed, "normally" developed fetuses were found in the uterus.

Figure 3: Spontaneous malformations in 3918 fetuses from 414 NMRI-control-mice. The mother animals were caused to die on the 19th day of gestation.
a. Type of malformation b. Number of malformed fetuses c. Frequency
d. Cleft palates e. Thoracic vertebral body hyplasty g. Aplasia of the vertebrae and ribs h. Malformations of the ribs i. Out of 50 mother animals.
f. execephaly. j. microcephaly k. micronathy

a. Art der Mißbildung	b. Zahl der mißgebildeten Feten	c. Häufigkeit (%)
d. Gaumenspalten	40	1,02
f. Exencephalie	6	0,15
j Mikrocephalie	2	0,05
k Mikrognathie	1	0,03
e Brustwirbelkörperhypoplasie	4	0,10
g Wirbel- und Rippenaplasie	1	0,03
h. Rippenmißbildungen	6	0,15
	60	1,53
	i von 50 Muttertieren	

Out of the 3918 living fetuses, 60 (1.53%), which originated from 50 (12%) of the 414 mother animals, exhibited spontaneous malformations (Table 3), and also 40 fetuses (1%) had cleft palates. Thus they presented the most frequent spontaneous malformation in our mice-breed. Four fetuses had execephaly, 2 fetuses had microcephaly, 1 fetus exhibited a micrognathy, and in 4 fetuses, hypoplasiae or aplasiae of some thoracic vertebrae were detected. Six fetuses had malformations of the ribs, and in one stunted animal, all the thoracic vertabrae, the ribs, and most of the lumber vertebrae were missing.

C. Results:

1. Testing the Fetotoxic Effect of Different Suspensions:

From the 11th to the 15th day of pregnancy, gravid mother-mice received daily 0.2 ml per animal of the suspension preparations (mentioned in Table 4) by intraperitoneal injection. The individual results are shown in Table 4.

Table 4: Effects of suspensions on the pregnancy of NMRI mice 5 x 0.2 ml/animal intraperitoneally as aqueous suspension from the 11th to the 15th day of gestation (The mother animals were caused to die on the 19th day of gestation)

a. Substance b. Number of mother animals c. Total number per group with miscarried fetuses d. Implantations e. Total number per group f. Average per mother animal g. Resorptions h. % referred to the total number of implantations i. Miscarriages j. Fetuses k. Alive l. Weight m. Length n. Dead o. Malformed p. Type of malformation q. Distilled water r. Ringer's solution 1. Cleft palate 2. Umbilical hernia 3. Malformation of the ribs 4. Aplasia of the Os interparietale.

a. Substanz	b. Muttertierzahl		d. Implantationen		g. Resorptionen		i. Aborte		j. Feten							
									k. lebend				n. tot	o. mißgebildet		
	c. ges.	mit mißgeb. Feten	ges.	0	h. ges.	%	h. ges.	%	h. ges.	0	l. Gewicht g 0	m. Länge cm 0	ges.	ges.	%	p. Art der Mißbildung
q. Aqua dest.	21	1	225	10,7	24	10.7	0	0	200	9.5	1.2	2.2	1	1	0.4	1 · 1
r. Ringerlösung	5	0	54	10,8	4	7.4	0	0	50	10.0	1.3	2.5	0	0	0	–
Gummi arabicum (1%)	8	1	92	11,5	3	3.3	0	0	88	11.0	1.3	2.2	1	1	1.1	1 · 2
Agar (1%)	9	2	94	10.4	9	9.6	4	4.3	81	9.0	1.2	2.2	0	4	4.3	3 · 1 1 · 3
Carrageen (1%)	9	1	92	10,2	9	9,8	0	0	82	9.1	1.2	2.2	1	1	1.1	1 · 1
Guar (1%)	11	0	113	10,3	42	37.2	9	8.0	52	4.7	1.1	2.1	10	0	0	·
Guar (1%) 5 × 0,1	8	0	72	9,0	12	16.7	2	2.8	55	6.9	1.1	2.1	3	0	0	·
Talkum (1%)	9	1	87	9,7	11	12,6	0	0	76	8.4	1.2	2.3	0	1	1.1	1 · 2
Traganth Nr. 0 (1%)	8	0	82	10,3	44	53.7	38	46.3	0	0	—	—	0	0	0	–
Gummi arabicum (10%)	18	5	202	11.2	57	28.2	0	0	145	8.1	1.2	2.3	0	6	3.0	3 · 1 3 · 4
Talkum (10%)	14	1	128	9.1	50	39.1	0	0	72	5.1	1.2	2.2	6	2	1.6	2 · 1

ges. = Gesamtzahl pro Gruppe, 0 = Durchschnitt pro Muttertier, % bezogen auf Gesamtimplantationszahl. 1 = Gaumenspalte 2 = Umbilicalhernie, 3 = Rippenmißbildung, 4 = Aplasie des Os interparietale.

After a 5 time intraperitoneal injection from the 11th to the 15th day of gestation of each time 0.2 ml per animal of distilled water, Ringer's solution, 1% aqueous gum arabic, agar and carrageen mucilage or of a 1% aqueous talc suspension, there was no disturbance of the pregnancy. After the injection of corresponding amounts of a 1% aqueous guar mucilage, one of the 11 mother mice died intercurrently. Forty-two fetuses were resorbed, 9 were expelled as miscarriages, and 10 were dead at the time of the killing of the mother animals

on the 19th day of gestation. In doses of 5 times 0.1 ml/animal, a 1% guar mucilage no longer influenced practically the fetal development.

After the 5 time intraperitoneal injection of each time 0.2 ml of a 1% aqueous mucilage of a commercially obtained Near East DAB 6 tragacanth, surprisingly all the fetuses were resorbed or were expelled as miscarriages. This ~~embryo~~ feto-toxic effect of the 1% aqueous tragacanth mucilage investigated was considerably stronger than that of a 10% aqeous gum arabic mucilage or of a 10% aqueous talc suspension.

2. Embryotoxic Effect of Persion Tragacanth:

Since the ~~embryo~~ feto-toxic effect (observed in the first test) of the investigated tragacanth specimen stood in contrast with all the other suspensions tested, and in order to exclude experimental errors or an accidental contamination of this specimen by unknown active substances, which could have caused the embryotoxic effect, 5 other charges (No. 1 - 5) of the DAB 6-tragacanth (of Persion origin) provided by the Merck Company and used in Test 1, as well as in each case one specimen of Persion tragacanth of the Imhoff & Stahl Company (No. 6) and of the Riedel de Haen Company (No. 7) were introduced into the investigations.

In these tests the animals were injected in each case with 0.2 ml of the 1% mucilages in distilled water only on the 11th and 12th day of gestation. Furthermore, some mice were treated on the 14th and 15th day of gestation with three different Persion tragacanth specimens in the corresponding way. The individual results are given in Figure 1.

Figure 1: Embryotoxic effect of Persion tragacanth for NMRI mice 2 x 0.2 ml/animal intraperitoneally as 1% aqeous mucilage. Resorptions, Miscarriages, Dead fetuses, Alive fetuses.

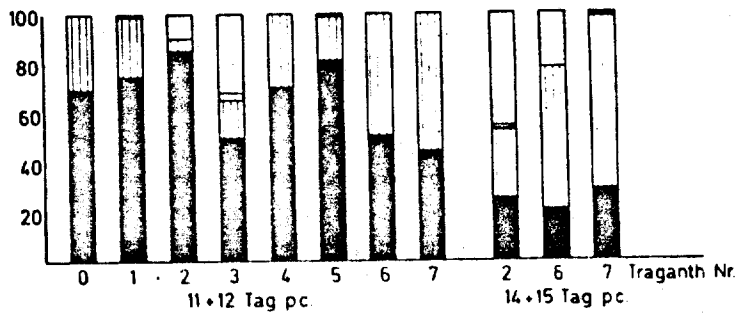

Abb. 1. Embryotoxische Wirkung persischen Traganths für NMRI-Mäuse 2 × 0,2 ml/Tier i.p. als 1%iger wäßriger Schleim. Resorptionen, Aborte.

Five hundred and twenty eight of the 818 fetuses of the 87 mother mice, into which each time 0.2 ml/animal had been injected intraperitoneally on the 11th and 12th day of gestation, were resorbed, and 255 were expelled as miscarriages. After injection on the 14th and 15th day of pregnancy, 12 of the 160 fetuses of the 15 mother animals were dead, 40 resorbed, and 82 were expelled as miscarriages.

The miscarriages, externally recognizable through vaginal bleedings, occurred in most cases 2 to 3 days after the treatment. While animals were being killed during vaginal bleeding, dead fetus residues were found in the uteri.

Thus all the tested charges of Persian tragacanth exerted a considerable embryo-toxic effect.

Since already an only two-time intraperitoneal injection of 0.2 ml each time of a 1% tragacanth mucilage of Persian origin, had a highly ~~embryo~~ feto toxic effect, a Persian tragacanth specimen of the three supplying Companies was injected into gravid mice only one intraperitoneally. The injections were conducted on the 11th, 12th or 14th day of gestation. See the individual results in Table 5.

Table 5: ~~Embryo~~ Feto toxic effect of Persian tragacanth for NMRI mice 1 x 0.2ml/animal intraperitoneally as 1% aqueous mucilage (The mother animals were caused to die on the 19th day of gestation)

a. Day of application percent b. Number of mother animals c. Total number per group
d. with born fetuses e. Implantations f. Average per mother animal
g. Resorptions h. % referred to the total number of implantations i. Miscarriages
j. Fetuses k. Alive l. Weight m. Length n. Dead o. Malformed
p. Type of malformation I = cleft palate 2 = Kyphosis 3 = Malformation of the thoracic vertebral body 4 = Umbilical hernia 5 = Clubfoot on the left aplasia of the metatarsals and phalanges III and IV.

Tabelle 5. *Embryotoxische Wirkung persischen Traganths für NMRI-Mäuse 1 - 0,2 ml/Tier i.p. als 1%iger wäßriger Schleim (Tötung der Muttertiere am 19. Trächtigkeitstag)*

Traganth Nr.	a Applikationstag p.c.	b Muttertierzahl		e Implantationen		g Resorptionen		Aborte		j Feten								
		c ges.	mit mißgeb. Feten	f ges.	0	f ges.	%	ges.	%	k lebend ges.	0	l Gewicht g 0	m Länge cm 0	n tot ges.	o mißgebildet ges.	%	p Art der Mißbildung
2	11	8	3	85	10,6	11	12,9	0	0	72	9,0	1,1	2,1	2	6	7,1	6 · 1
6	11	10	0	104	10,4	58	55,8	13	12,5	27	2,7	1,0	2,1	6	0	0	
7	11	6	0	64	10,7	46	71,8	9	14,1	9	1,5	1,2	2,3	0	0	0	
0	12	15	5	150	10,0	45	30,0	0	0	105	7,0	1,2	2,2	0	10	6,7	9 · 1 1 ·
2	12	11	5	116	10,5	17	14,7	5	4,3	93	8,5	1,1	2,2	1	14	12,1	13 · 1 1 ·
6	12	8	2	81	10,1	55	67,9	0	0	22	2,8	1,0	2,0	4	3	3,7	3 · 1
7	12	8	0	84	10,5	22	26,2	62	73,8	0	0	0	0	0	0	0	
0	14	5	0	45	9,0	41	91,1	4	8,9	0	0	0	0	0	0	0	
2	14	3	2	30	10,0	5	16,7	0	0	24	8,0	1,1	2,2	1	3	10,0	3 · 1
6	14	8	2	90	11,3	20	22,2	0	0	22	2,8	1,0	2,1	48	7	7,8	5 · 1 1 · 1 · 2
7	14	5	0	52	10,4	0	0	31	59,6	4	0,8	1,0	2,1	17	0	0	

ges. = Gesamtzahl pro Gruppe. 0 = Durchschnitt pro Muttertier. % = bezogen auf Gesamtimplantationszahl. 1 = Gaumenspalte 2 = Kyphose, 3 = Brustwirbelkörpermißbildung, 4 = Umbilicalhernie, 5 = Klumpfuß links Aplasie der Metatarsalia und Phalange III und IV.

Abb. 2. Links: Fet mit hypoplastischer Fehlbildung der hinteren Extremität und Tibiaaplasie. Behandlung der Muttermaus: einmal 0,2 ml pro Tier i.p. eines 1%igen wäßrigen Schleims aus persischem Traganth am 12. Trächtigkeitstag. Rechts: Normaler Kontrollfet. Muttermäuse wurden am 19. Trächtigkeitstag getötet. Aufhellung und Anfärbung des Skeletsystems nach einer modifizierten Dawson-Methode

In these tests, the strongest ~~embryo~~ feto toxic effect was exerted by Charge No. 7, but even after injection of the tragacanth-Charge No 6 on the 11th or 12th day of gestation, the majority of the fetuses were resorbed or expelled as miscarriages.

After the injection of this tragacanth (No. 6) on the 14th day of gestation, 20 fetal resorptions, 22 alive and 48 dead fetuses were found in the uteri of the 8 mother mice treated. One of the 22 living, and 4 of the 48 dead fetuses had cleft palates. Furthermore, kyphosis was detected in a dead fetus, and in another a malformation of the thoracic vertebral body. The seven malformed living and dead fetuses originated from two of the eight mother mice.

The Persian tragacanth specimens No. 0 and 2, used for these tests, produced fetal resorption and abortions with lower frequency; nevertheless, 29 of the 294 living fetuses and 2 of the dead ones had cleft palates. The 10% proportion of cleft palates is 10 times higher than the spontaneous cleft palate frequency (1.02%) of our NMRI mice-breed. The 29 living and 2 dead fetuses with cleft palates come from 14 different mother animals.

Cleft palates occurred most frequently in the mother mice treated on the 12th day of pregnancy with Persian tragacanth mucilage No. 0 and No. 2; then in 10 of the 26 mother animals, fetuses (21 living and 1 dead) were found with a fissure of the palate. Furthermore, one fetus of these 10 mother animals had an ectopia of the abdominal organs. In another fetus with uranoschism, once the skeleton had been colored, a hypoplastic malformation of the right rear extremity with tibial aplasia was detected (Figure 2). Thus altogether in 10 of the 26 mother mice treated on the 12th day of gestation, 23 living fetuses and one dead, were found with malformations.

Figure 2: Left: Fetus with hypoplastic malformation of the read extremity and tibial aplasia. Treatment of the mother mouse: one 0.2 ml per animal intraperitoneally of a 1% aqueous mucilage from Persian tragacanth on the 12th day of gestation. Right: Normal control fetus. The mother mice were caused to die on the 19th day of pregnancy. Clarification and coloration of the skeletal system by a modified Dawson method.

A 1% aqueous mucilage of Persian tragacanth of different origin(s) exerted an embryotoxic effect not only after the repeated, but already after the single intraperitoneal injection of 0.2 ml per animal in the middle of the gestation in the case of the NMRI mice used for the investigations.

In order to determine whether tragacanth from other regions of cultivation possesses the same embryotoxic effect as Persion tragacanth, two different specimen of Indian tragacanth (No. 1 and 2) were tested in the same way as in Experiment 1.

Unlike Persian tragacanth, these Sterculia-rubber specimens, even after the five-time injection of 0.2 ml per animal from the 11th to the 15th day of gestation - a dose which in the case of the Persian tragacanth always caused 100% fetal death - failed to affect the fetal development of the mice. Only the proportion of resorptions was slightly raised as compared with the normal occurrence, and 7 of the 239 fetuses (2.9%), from 7 different mother animals, had uranochism.

The repeated five time subcutaneous injection of each time 0.2 ml per animal from the 11th to the 15th day of gestation of a 1% aqueous mucilage, obtained from Persian tragacanth No. 2, failed to affect the fetal development of all the 12 mother mice.

Hence, it must be assume that the embryotoxic effect (observed after intraperitoneal injection) of different tragacanth charges of Persian origin is conditioned not by a systemic, but by a direct action on the uterus.

Since some of the investigated suspensions are used also in manufacturing suspensions to be orally applied, and since tragacanth as well as guar and agar are allowed as food thickeners, the fetotoxic effect of these natural substances, as compared with gum arabic and talc, was tested by oral application also on 35 mice. The soundings (probings) were undertaken from the 11th to the 15th day of gestation daily in amounts of 0.5 ml per animal in the form of 1% and 10% suspensions or mucilages. Despite this high dosage, the proportion of fetal resorption, the average number of living fetuses,

their body lengths and weights as well as the malformation rate in the tests with Persian and Indian tragacanth, agar and gum arabic, lay within normal limits. It is only after the sounding of 10% talc suspension and of 1% guar mucilage that the proportion of fetal resorption was raised.

Thus Persian tragacanth in oral application of large amounts even, like the other natural substances tested, failed to affect the fetal development of the NMRI mice.

3. Embryotoxic Effect of Suspensions in Early Pregnancy:

Previous tests with chemical, teratogenic compounds like formamide, monomethyl formamide, 1-ethylene imino-2-oxybutene-3 and 6-mercaptopurine showed that during the early period of gestation the mice-embryos were only slightly sensitive to teratogenic noxas (OETTEL and FROHBERG, 1964/1965). Hence tests were conducted in order to determine whether the embryotoxic effect of Persian tragacanth after intraperitoneal injection during the first third of the gestation is weaker than after application between the 11th and 15th day of gravidity.

Sixteen mice were injected from the 4th to the eighth day of gestation with 0.2 ml of a 1% aqueous mucilage from Persian tragacanth intraperitoneally. For comparison purpose, mice were treated in the same way with distilled water or mucilages from different natural substances (agar, guar, gum arabic, Indian tragacanth). Like in the previous tests, the animals were caused to die on the 19th day of gestation, that is one day before the normal littering term.

In all the 31 mice treated with distilled water, agar, gum arabic or Indian tragacanth - apart from the 17.6% amount of resorption some what increased in the "agar animals" as compared with what is normal - the fetal development was not disturbed. The number of fetuses, their weight and length, the proportion of malformations and resorptions corresponded to those of the untreated mice. On the other hand, normally developed fetuses were found only in one of the 16 animals treated with Persian tragacanth. The uteri of the other 15 mother mice, when these were killed on the 19th day of gestation did not differ from those of nonpregnant animals.

The same dose, administered on the 6th and 7th day of gestation, caused vaginal bleedings in 3 of the 7 mother animals. Hence, these mice, and also those treated on the 7th and 8th day of gravidity, were caused to die not one day before littering, but already on the 12th day post coitum. At this moment, fetuses (66) still living in the uteri were found only in 6 of the 13 mice. In 4 of the 13 mothers, all the fetuses had been expelled. It was possible, however, to determine the number of the expelled embryos by observing the places of implantation that were still visible - in the form of "metrial glands".

On the other hand, in 6 out of 16 mice, which had been treated from the 4th to the 8th or from the 4th to the 7th day of gestation with tragacanth mucilage and had been killed immediately, the uteri did not differ from those of nonpregnant mice. Since, however, in 2 of the 16 animals, normal embryos, corresponding in their development to the 7th or 8th day of gestation, were present in the uteri, and since in the other mice the places of implantation were clearly recognizable on the mesometrium insertion, it must be assumed that in the 6 animals without "metrial glands" the embryos were expelled already before, or at the beginning of, the embedding process.

Thus the embryotoxic effect of Persian tragacanth was weaker in the case of injections administered during early gestation than in application in the middle of the gravidity period. As is shown by the tests, the mice-embryos with increasing age become more sensitive to the embryotoxic effect of Persian tragacanth; whereas Persian tragacanth mucilage injected on the 4th and 5th day of gestation failed to disturb the embryo development, fetal death and miscarriage or resorption occurred after injection on the 6th and 7th day of gestation, and still more markedly after application on the 7th and 8th day of gravidity.

4. Comments on the Problem of the Antineoplastic Effect of Suspensions:

ROE (1959), GALBRAITH, MAYHEW and ROE (1962) as well as MAYHEW and ROE (1964) found that native Persian tragacanth, but not heated Persian tragacanth and Karaya rubber (= Indian tragacanth) inhibited the growth of mice-ascites-tumors.

On the basis of mitosis counts on the Landschütz-ascites-tumor of the mice, MAYHEW and ROE (1964) surmized that the cause of the antineoplastic effect (observed by them) of the native true tragacanth was a mitosis inhibition and an "active component" of this drug, influencing the surface of the tumor cells. Hence tests were conducted in order to determine whether the embryotoxic effect, observed in the case of the Persian tragacanth, was an expression of the cytotoxic action described by ROE and Collaborators for tragacanth. For this purpose, Persian tragacanth (No. 0) was subjected to experimentation regarding antineoplastic action on the Ehrlich-ascites-carcinoma of the mouse, as compared with other suspensions and natural substances.

For each series of tests, 40 to 60 mice were used. All the animals were inoculated intraperitoneally with fresh ascites 1:1 or 1:10 diluted with Ringer's solution. Each test or control group comprised 10 mice; the controls remained untreated. All experimental animals - starting two hours after the tumor transplantation - were inoculated intraperitoneally on 5 consecutive days with 0.2 ml per animal of distilled water or of a 1% aqueous suspension of the corresponding suspensions. The mice were weighed daily, they were observed until their spontaneous death; then they were dissected. The ascites was removed by sponging the abdominal cavities with cellulose, and its amount was determined by subsequently weighing the animals. The evaluation of the antineoplastic effect was made on the basis of the ascites amount found, and also of the surviving period of the tumor animals (OETTEL and WILHELM, 1957). See individual results on Table 7.

Table 7: Antineoplastic effect of natural substances on the Ehrlich-ascites-carcinoma of the mouse. Administration of 5 x 0.2 ml/animal intraperitoneally of a 1% aqueous suspension (Monday until Friday). (First injection two hours after the tumor transplantation).

a. Substance b. Life duration in days c. Average value d. Absolute range of dispersion e. Amount of ascites at death f. Tumor inhibition in % g. Test series I h. Untreated i. Distilled water j. Gum arabic 10% k. Talc 10% l. Indian tragacanth m. Persian tragacanth o. agar (threads) p. Carrageen q. Guar

In the tumor transplantation, 1 ml of diluted fresh ascites was injected intra peritoneally into the mice. Ringer's solution was used for dilution. Dilution ration 1:1 in the series of tests I, and 1:10 in II and III.

Tabelle 7. *Antineoplastische Wirkung von Naturstoffen am Ehrlich-Ascites-Carcinom der Maus. 5 × 0,2 ml/Tier i.p. einer 1%igen wäßrigen Suspension (Montag bis Freitag). (Erste Injektion 2 Std nach der Tumortransplantation)*

a Substanz	b Lebensdauer in Tagen		e Ascites-menge beim Tod (g)	f Tumor-hemmung in %
	c Mittel-wert	d Absolute Streuung „range"		
g *Versuchsreihe I*				
h Unbehandelt	11,9	8—15	9,6	—
i Aqua dest.	11,5	7—15	9,1	4,4
j Gummi arabicum 10%	8,2	6—12	4,1	57,6
k Talkum 10%	9,1	7—12	5,2	45,3
l Ind. Traganth (Nr. 2) 1%	9,0	8—15	5,0	47,5
m Pers. Traganth (Nr. 0) 1%	6,8	4—8	1,6	83,2
Versuchsreihe II				
Unbehandelt	14,9	12—18	9,3	—
Aqua dest.	12,8	7—17	8,0	13,7
Gummi arabicum 10%	11,3	9—14	5,1	45,1
Talkum 10%	10,7	4—15	4,4	52,5
Ind. Traganth (Nr. 1) 1%	10,2	8—13	4,2	54,4
Pers. Traganth (Nr. 0) 1%	8,1	5—14	0,5	94,7
Versuchsreihe III				
Unbehandelt	11,6	9—15	6,2	10,5
n Agar (Fäden) 1%	9,1	8—13	2,0	67,7
o Carrageen 1%	11,0	8—16	5,6	10,5
p Guar 1%	9,1	8—12	2,8	55,2

Bei der Tumortransplantation wurde den Mäusen jeweils 1 ml verdünnter, frischer Ascites i.p. injiziert. Zur Verdünnung wurde Ringerlösung verwandt. Das Verdünnungsverhältnis betrug in der Versuchsreihe I 1:1, in den Versuchsreihen II und III 1:10.

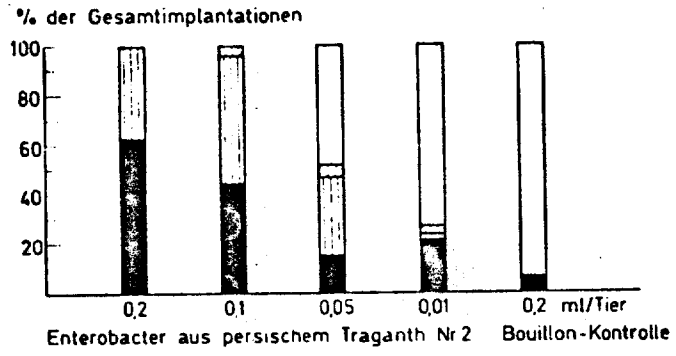

Abb. 5. Embryotoxische Wirkung kleinerer Dosen von Enterobacter-Bouillon-Sterilfiltraten für NMRI-Mäuse einmal i.p. am 12. Tag p.c. ■ Resorptionen, ▭ Aborte, ▭ Mißbildungen, ▭ lebende Feten

With the exception of the carrageen animals, in all the series of tests, in the mice treated with the different suspensions, the ascites amount decreased by 50% as compared with the controls, and even by 80 to 90% in the animals treated with Persian tragacanth. This decrease of the ascites amount, however, implied in every case the shortening of the surviving period of the experimental animals as compared with the controls, and thus does not express a true tumor inhibition.

5. Effects of Heat Sterilization and of Antibiotics on the Embryotoxic Action of Persian Tragacanth:

In trying to find the causes of the embryotoxic effect of Persian tragacanth, tests were performed in order to ascertain whether this effect was due possibly to microbial contaminations. For this purpose, a specimen of Persian tragacanth (No. 2) and for comparison a specimen of Indian tragacanth (No. 1) were dry-sterilized 30 minutes at 170 C. Then, from these two tragacanth specimens and with distilled water in each case a 1% mucilage was manufactured which was used in treating intraperitoneally mice from the 11th to the 15th day of gestation. Furthermore, mother mice were treated in the same way with an aqueous mucilage from Persian tragacanth which had been previously incubated for 48 hours at 37 C after adding penicillin (100 I.U./ml) and streptomycin (100 μg/ml) (See Figure 3).

Figure 3: Embryotoxic effect of pretreated Persian tragacanth for NMRI mice 5 x 0.2 ml/animal intraperitoneally as 1% aqueous mucilage from the 11th to the 15th day p.c.(?) Resorptions Miscarrages Dead fetuses Living fetuses

a. % of the total implantations b. Untreated c. Antibiotics d. Sterilized

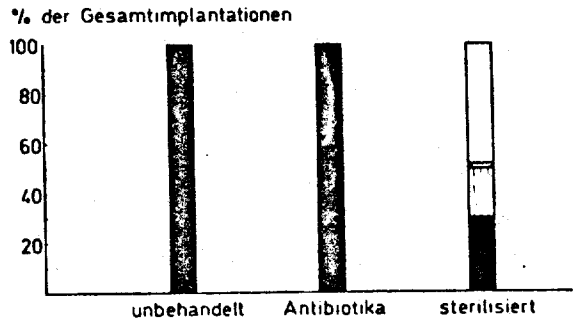

Abb. 3. Embryotoxische Wirkung vorbehandelten persischen Traganths für NMRI-Mäuse 5 × 0,2 ml/Tier i.p. als 1%iger wässriger Schleim vom 11.–15. Tag p.c. Resorptionen, Aborte, tote Feten, lebende Feten

Through a 30 minute sterilization at 170 C, the embryotoxic effect of Persian tragacanth was decreased, since even after the five time injection of this mucilage, manufactured from sterilized true tragacanth, only half the fetuses were resorbed or were expelled as abortions. As was expected, heat-sterilized Indian tragacanth influenced the fetal development just as little as untreated (Indian tragacanth).

A penicillin and streptomycin addition failed to lower the embryotoxic effect of Persian tragacanth.

6. Bacteriological Investigation of the Different Tragacanth Specimens:

Since it was possible to lower the embryotoxic action of Persian tragacanth through heat sterilization, it was presumable that microorganisms or their products of metabolism might be the cause of the embryotoxic effect of Persian tragacanth. Hence, the different charges of Persian and Indian tragacanth were examined bacteriologically.

In order to isolate the microorganism contained in the individual tragacanth charges, 1% mucilage specimens were prepared under sterile conditions with sterile physiological salt solution. From these specimens, after a 48 hour incubation at 37 C, a loop was inoculated on nutrient agar and the dishes were incubated at 37 C for 24 to 48 hours. Furthermore from the different 1% mucilage specimens, 1 ml in each case was placed in 50 ml of nutrient broth, and thus was processed in the same way.

In order to determine the presence of possible impurites through fungi, the same tragacanth specimens were inoculated also on Sabouraud nutrient media and Sabouraud broth, and were incubated for 14 days at 22 C.

From the germs, grown on the agar dishes and in the broth cultures, pure cultures were obtained, and the different types of germs were examined morphologically, biochemically, and in the case of the gram negative bacteria serologically also (specimen agglutination with salmonella cumulative serum I-Behring Plant).

In order to obtain an accurate determination of the isolated gram negative germs, the following properties were tested regularly, or the following reactions were conducted:

Motility
Hemolysis
Gelatin liquefaction
H_2S formation
Urea dissociation (splitting)
Methyl red reaction
Voges-Proskauer reaction
Indole reaction
Koser citrate
"Multicolored Series" with the following hydrocarbons and alcohols: dextrose, lactose, saccharose, salicin, inositol, adonitol, dulcitol.

For further differenciation, in individual cases, the following culture characteristics were investigated also:

Splitting of sorbitol, xylose, arabinose, starch.
Behavior in litmus milk, nitrate reduction, detection of Phenylalanine-decarboxylase, lysine-decarboxylase, catalase, cytochrome-oxydase.

In the case of the gram positive bacilli, the following culture characteristics were used for diagnostic purpose:

Growth in broth and on agar
Motility
Hemolysis
Gelatin liquefaction
Splitting of dextrose, lactose, saccharose, xylose, mannitol
Indole reaction
Voges-Proskauer reaction.

In the case of the isolated gram positive diplococci, the following investigations were conducted:

Growth in broth and on agar
Hemolysis
Splitting of dextrose, saccharose, lactose, mannitol, maltose, salicin, sorbitol, induline, aesculine
Indole reaction.

On the basis of the undertaken investigations, the germs, isolated out of the different tragacanth specimens, were determined as follows: Results in Table 8.

Table 8: Bacteriological findings relative to different tragacanth specimens
a. Tragacanth specimen b. Yeasts c. Fungi

a. Traganth-probe	b. Bakterien		c. Hefen Pilze
	gramnegativ	grampositiv	
Pers. Traganth			
1	Enterobacter aerogenes	—	—
2	Enterobacter aerogenes	—	—
3	Enterobacter aerogenes	—	Penicillium
4	Enterobacter aerogenes	—	—
5	Enterobacter aerogenes	—	—
6	Enterobacter aerogenes Flavobact. rhenanus	Bac. circulans Bac. subtilis Diplococcus (nicht differenziert)	Mucor
7	a) Enterobacter aerogenes b) Enterobacter aerogenes	Bac. megatherium Diplococcus (nicht differenziert)	—
Ind. Traganth			
1	—	Bac. mesentericus Bac. subtilis	— —
2	—	Bac. mesentericus Bac. subtilis	—

After these investigations, enterobacter aerogenes germs were detectable in all the Persian tragacanths, and even as pure culture in specimens 1 to 5.
In the gragacanth specimen No. 6, a flavobacterium rhenanus, belonging to the family of the achromobacteraceae, was still isolated. In the tragacanth specimens

In the case of the isolated gram positive diplococci, the following investigations were conducted:

Growth in broth and on agar
Hemolysis
Splitting of dextrose, saccharose, lactose, mannitol, maltose, salicin, sorbitol, induline, aesculine
Indole reaction.

On the basis of the undertaken investigations, the germs, isolated out of the different tragacanth specimens, were determined as follows: Results in Table 8.

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7	a) Enterobacter aerogenes b) Enterobacter aerogenes	Bac. megatherium Diplococcus (nicht differenziert)	—
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1	—	Bac. mesentericus Bac. subtilis	— —
2	—	Bac. mesentericus Bac. subtilis	—

After these investigations, enterobacter aerogenes germs were detectable in all the Persian tragacanths, and even as pure culture in specimens 1 to 5.
In the gragacanth specimen No. 6, a flavobacterium rhenanus, belonging to the family of the achromobacteraceae, was still isolated. In the tragacanth specimens

No. 6 and 7, it was possible to detect, in addition to enterobacter-aerogenes germs and the flavobacterium, gram positive aerobic sporiferous (bacteria ?) as well as gram positive diplococci, which were not more closely differentiated. In the tragacanth specimens No. 3 and 6, a penicillium or a Mucor germ was detectable; the latter was not included in the further investigations.

In contrast with the results of bacteriological investigations, in the Persian tragacanth specimens of different origins, no germs had grown in the parallel text with the two Indian tragacanths after an incubation of 24 hours. It was only after a storage of 36 to 48 hours at + 37 C, that on the nutrient agar dishes different gram positive aerobic sporiferous bacteria (Bac. subtilis, Bac. mesentericus were detected. The only low number of bacteria in the Sterculia rubber should be attributed to the fact that the commercial product is solubilized through the autoclave (Merck Index, 8th Edition 1968).

According to the investigations conducted so far, the gram negative germs contained in the Persian tragacanth specimens might be regarded as the cause of the embryotoxic effect. Hence, tests were performed in order to determine the possibility of isolating these gram negative bacteria from the absominal cavity, after repeated intraperitoneal tragacangh injection.

For this purpose, two mice, which had been treated intraperitoneally one, 3 or 5 times with 0.2 ml of a 1% mucilage of Persian or Indian tragacanth, were caused to die nearly 8 hours after the last injection; the abdominal cavity was opened under sterile conditions, the peritoneal fluid between the partly adherent intestinal loops were removed, and inoculated on agar dishes.

It was possible to isolate the enterobacter germs, detected bacteriologically in the different Persian tragacanth specimens - although these germs were few - from the absominal cavity of the mice treated intraperitoneally 4 or 5 times with these mucilage specimens. In the test with Persian tragacanth No. 6, the gram positive diplococci were found again in the abdominal cavity of the animals treated with this tragacanth. Nevertheless, it was impossible to isolate the gram positive aerobic sporiferous bacteria, primarily detected in the Persian tragacanth specimens No. 6 and 7 or in the Indian tragacangh No. 1, from the

abdominal cavity of the mice treated repeatedly intraperitoneally in the case of these specimens.

7. Embryotoxic Effect of Products of Metabolism of Bacteria from Persian Tragacanth:

In order to exclude a bacterially induced peritonitis as cause of the embryotoxic effect of Persian tragacanth, tests were conducted in order to ascertain whether products of metabolism of the isolated germs can also exert an embryotoxic effect. For this purpose, normal nutrient broth was inoculated with pure cultures of the gram negative germs isolated from the different Persian tragacanth specimens (2 Pt loops of a 24-hour culture on 50 ml of broth) and was incubated during 48 hours at +37 C. Then these broth cultures were passed under sterile conditions through bacteria-proof filters (Seitz/Filter - Layer EKS II (EKS = sterilization layer)). After verifying the sterility by preparing smears on agar dishes, these broth cultures made sterile by filtration were injected under sterile conditions with the products of metabolism of the enterobacter strains in mice intraperitoneally - starting on the 11th day of gestation - in doses of 0.2 ml per animal (Figure 4).

Figure 4: Embryotoxic effect of broth cultures, filtrated under sterile conditions, of enterobacter strains for NMRI mice. 1 x 0.2 ml/animal intraperitoneally on the 11th day of gestation Resorptions, Miscarriages, Dead fetuses, Living fetuses, Malformations.

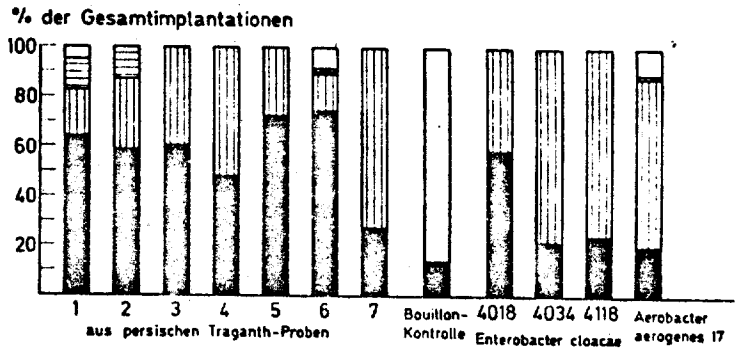

Abb. 4. Embryotoxische Wirkung steril filtrierter Bouillon-Kulturen von Enterobacter-Stämmen für NMRI-Mäuse. 1 × 0,2 ml/Tier i.p. am 11. Tag p.c. ■ Resorptionen, ▤ Aborte, ▩ tote Feten, ▭ lebende Feten, ▭ Mißbildungen

Already in the course of 12 to 15 hours after the first intraperitoneal injection, vaginal bleedings - which must be regarded as sign of miscarriage - developed in 7 of the experimental groups, which had been treated with enterobacter broth culture filtrates from the Persian tragacanth specimens No. 1 to 7, in the majority of the mice. Hence, the mice of these 7 experimental groups, which for a certain time (1 - 2 days) exhibited a ruffled skin and retracted flanks, were treated only once. Three mother animals died intercurrently.

Despite the only single intraperitoneal injection of the Persian tragacanth specimens No. 1 - 7, living fetuses were found in uteri only in 2 of the 55 mother mice treated. All the other 543 fetuses had been resorbed or had been expelled as abortions.

The 13 mice of the two other experimental groups developed no vaginal bleedings, hence they were treated from the 11th to the 15th day of gestation. In this experimental setup, the two sterile filtrates, which contained products of metabolism of the flavobacterium rhenanus and of an enterobacter aerogenes, exerted an embryotoxic effect, since about half the fetuses of the mother animals were rsorbed or expelled as miscarriage. The embryotoxic action of the products of metabolism of this flavobacterium rhenanus and of this enterobacter strain, however, was considerably weaker than that of the other enterobacter strains, which already after the single injection practically caused the death of all the fetuses. In its cultural properties also, the enterobacter germ differed from those of the other enterobacter strains.

These investigations reveal that the sterile filtrates of broth cultures, inoculated with enterobacter germs and incubated for 48 hours at +37 C, exerted an embryotoxic effect.

In order to determine whether other strains of the same genus had an embryotoxic effect, sterile filtrate of broth cultures of the aerobacter aerogenes strain 17 (Dr. F. Selenka) and of 3 enterobacter cloacae strains (Dr. B. Schmidt) were tested (The long footnote at this point merely expresses thanks to these two university lecturers).

The sterile filtrates of the enterobacter strains defined caused the death of 241 out of 253 fetuses of the 34 mother mice, after a single injection on the 11th day of gestation (See Figure 5 p. 14a).

A 1% aqueous mucilage from Persian tragacanth, after repeated subcutaneous injection, had no embryotoxic effect. Hence, a broth culture made sterile by filtration was prepared from the same gragacanth charge in the way described above. This filtrate was injected under sterile conditions subcutaneously in 18 mice from the 11th to the 15th day of gestation in doses of 0.2 ml/animal. Furthermore 5 gravid mice were treated in the corresponding way with a broth culture made sterile by filtration; this filtrate had been prepared with enterobacter cloacae 4034. The 8 control mice received individually 0.2 ml of empty broth.

The fetal development of the 23 mother animals, treated with sterile filtrates of broth culture (obtained from Persian tragacanth No. 2 and from enterobacter cloacae 4034), did not differ from the 8 control mice treated with empty broth.

These "subcutaneous tests" corroborated the assumption that the embryotoxic effect (observed after intraperitoneal injection in NMRI mice) of tragacanth contaminated with bacteria of the genus enterobacter, is due not to a systemic but to a direct action on the uterus and on its vacular system.

In the tests reported at the beginning of this study, a single intraperitoneal injection of a 1% Persian tragacanth mucilage was followed, especially on the 12th day of gestation by increased malformations and especially by cleft palates (See Table 6). Hence, tests were conducted in order to determine whether the products of metabolism of enterobacter in doses, which practically no longer killed all the fetuses or produced resorption or miscarriage, possibly caused increased malformations.

For this test, a sterile filtrate, which had been obtained from a broth incubated for more than 48 hours with enterobacter strain 2, was injected in gravid mice in doses of 0.2; 0.1; 0.05 and 0.01 ml per animal once on the 12th day of gestation.

Like in the previous tests, on the 11th day of gestation, after a single intraperitoneal injection on the 12th day of gestation of 0.2 ml/animal of the sterile filtrate of broth culture, practically all the fetuses were resorbed or were expelled as miscarriage. The two surviving fetuses of the "0.1 ml group" had uranoschism. After the injection of 0.05 ml/animal, about half of the fetuses were killed, and even after the administration of only 0.01 ml/mouse still 16 of the 75 fetuses were resorbed, and 2 were expelled as miscarriage. Four of the 79 living fetuses of these two series of tests had malformations, especially cleft palates and some malformations of the extremities with extended subcutaneous bleedings.

In order to exclude the possibility for products of metabolism of the gram positive germ found in the different tragath specimens to possess an embryotoxic action, broth cultures made sterile by filtration were prepared under the same conditions as in the case of the gram negative germs from the different gram positive aerobic sporiferous bacteria (Bac. mesentericus and subtilis) and diplococci, which had been isolated from Persian and Indian tragacanth; and these cultures were used in injecting intraperitoneally mice from the 11th to the 15th day of gestation with 0.2 ml/animal daily.

In the same way, sterile filtrates of broth cultures of different other germs, stemming from the collection of the Industrial Hygiene Pharmacological Institute, such as E. coli I, Staph. aureus SG 511, Bac. subtilis and Bac. mesentericus, were tested.

Neither the sterile filtrates of broth cultures of the gram positive germs isolated from Persian and Indian tragacanth, nor the concomitantly tested control filtrates of E. coli, Staph. aureaus SG 511, Bac. subtilis and Bac. mesentericus possessed an embryotoxic action on the 103 gravid NMRI mice used for these tests.

8. Effect of the Use of the Autoclave on the Embryotoxic Action of Sterile Filtrates of Enterobacter Broth Cultures:

In order to find out whether through the use of the autoclave the embryotoxic action of products of metabolism contained in the sterile filtrates of broth cultures of enterobacter strains is decreased or eliminated, sterile filtrates of broth cultures of the strains Aerobacter aerogenes strain 17, Enterobacter cloacae 4034, Enterobacter strain from Persian tragacanth No. 2 and Enterobacter strain from Persian gragacanth No. 6 were subjected to a single treatment for 15 minutes at 120 C in the autoclave. These sterile filtrates of broth culture, thus treated, were injected once daily intraperitoneally in gravid mice once 0.2 ml/ animal on the 11th day of gestation or from the 11th to the 15th day of gestation in amounts of 0.2 ml/ animal (See Figure 6).

Figure 6: Embryotoxic effect of broth cultures (obtained by sterile filtration) from Enterobacter strains after the use of the autoclave for NMRI mice.

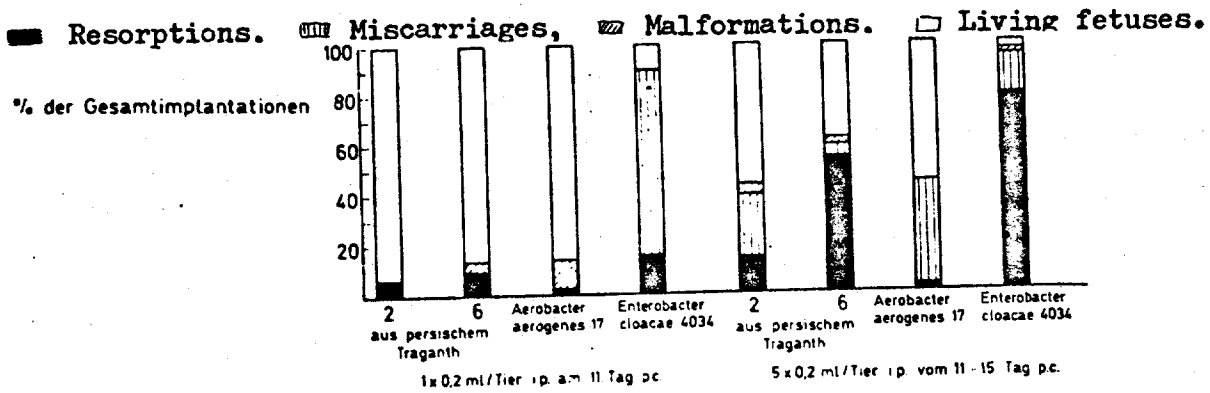

Abb. 6. Embryotoxische Wirkung steril-filtrierter Bouillon-Kulturen von Enterobacter-Stämmen nach Autoklavierung für NMRI-Mäuse. ■ Resorptionen, ▨ Aborte, ▭ Mißbildungen, ▭ lebende Feten

The use of the autoclave for 15 minutes weakened the embryotoxic effect of the products of metabolism of the different enterobacter strains, but failed to suppress it; after the five time intraperitoneal injection, about the half of the fetuses of the mother animals treated were resorbed or were expelled as miscarriages. The autoclaved sterile filtrate of the broth culture from Enterobacter cloacae 4034 was just as embryotoxic as the corresponding nonautoclaved sterile filtrate, since after the single injection on the 11th gestation day 77 out of 104 fetuses were expelled as abortion and 16 were resorbed.

Discussion:

Distilled water, Ringer's solution, the 1% mucilages of gum arabic, carrageen or guar, used in manufacturing suspensions or emulsions, and even 1% aqueous talc suspensions do not influence the fetal development of NMRI mice after a single intraperitoneal injection, or after several, in the sensitive phase of gestation. Under the same experimental conditions, however, once mother mices have been treated with 1% aqueous mucilages from 8 different Persian tragacanth specimens, the death and subsequent resorption or expulsion of all the fetuses occur. On the other hand, mucilage from 2 Indian tragacanth specimens exerts no effect, like the other natural substances tested, on the fetal development.

The mice-fetuses most easily injured are those involved in the treatment administered in the middle of the gestation period (11th and 12th day of gravidity). In the stage of advanced organogenesis, the fetuses become increasingly more resistant to the embryotoxic effect of Persian tragacanth. Thus the sensitivity of mice-embryos to pure tragacanth, during their development, is just as different as to known chemical or physical teratogenic noxa (WILSON, 1965; RUGH and GRUPP, 1959 as well as OETTEL and FROHBERG, 1965, 1965).

In all the mother mice, treated with a 1% aqueous Persian tragacanth mucilage intraperitoneally, dissection revealed inflammatory changes in the abdominal cavity. Hence at first it was assumed that the peritonitis, produced by the intraperitoneal injection, was the cause of the fetal injury, especially other natural substances, which do not affect the fetal development, like the investigated Indian tragacanth-, agar, or carrageen specimens which even after repeated intraperitoneal injection in the form of 1% aqueous mucilages do not exert a stimulating effect. There is an objection, however, against peritonitis as cause of the embryotoxic effect of Persian tragacanth: 1% aqueous gum arabic and especially talc suspensions after repeated intraperitoneal injection in mice cause stronger inflammatory stimulations in the abdominal cavity than Persian tragacanth, although these suspensions have just as little effect on the fetal development as Ringer's solution or distilled water. Even through five time intraperitoneal injection of a 10% aqueous gum arabic or talc suspension, the fetal development , despite most severe peritonitic inflammations and massive adherences of the ingesta in the mother animals is less disturbed than through

a single injection of 1% mucilage from Persian tragacanth. Hence, the peritoneal stimulation produced by this tragacanth after intraperitoneal injection should not be the cause of its embryotoxic action.

In contrast with the intraperitoneal tests, Persian tragacanth mucilage in oral application even after repeated administration of large amounts, affects the fetal development of mice just as little as the other tested natural substances. Hence - as far as a posteriori conclusions can be drawn from animal tests and applied to human beings - there should be no hesitation, from the embryotoxic effect standpoint, to continue the utilization of Persian tragacanth, beside other natural substances, as an addition to food.

A cytotoxic effect, described by ROE (1959), GALBRAITH, MAYHEW and ROE (1962) as well as MAYHEW and ROE (1964) in the case of Persian tragacanth on the basis of investigations with some mice-ascites-tumors, must be excluded, according to the investigations presented here, as cause of the observed embryotoxic effect of pure tragacanth, since the Persian tragacangh specimens, that we tested, like the other natural substances, possess on the Ehrlich ascites carcinoma of the mouse no significant antineoplastic effect after intraperitoneal injection.

According to our investigations, it is rather bacteria or their products of metabolism that must be made responsible for the embryotoxic effect of the Persian tragacanth charges tested. This is shown already by the decrease of the embryotoxic action of aqueous mucilage from Persian tragacanth after sterilization, and especially by the presence of gram negative coccoid rods of the genus Enterobacter in all the 7 investigated Persian tragacanth specimens, which are not found in the two Indian tragacanth specimens. The considerable lack of bacteria in Sterculia-rubber should be attributed to the solubilization of this commercial product through the autoclave (Merck Index, 1968). The products of metabolism of the individual isolated Enterobacter strains, injected intraperitoneally in the form of sterile filtrates of broth cultures in gravid animals, exert a higher embryotoxic effect than the original tragacanth mucilage. The products of metabolism of bacteria of these Enterobacter strains should represent thus the real active form. The control tests, conducted with defined Enterobacter strains, show that the embryotoxic effect is specific for the bacteria products of metabolism of the isolated bacteria genus (Enterobacter).

The gram positive aerobic sporiferous bacteria and diplococci, isolated from Persian tragacanth, generate no products of metabolism with embryotoxic action. In the same way behave also the gram positive germs, which were isolated from the Indian tragacanth specimens, or had been borrowed from the collection of our Institute. The broth culture, filtrated under sterile conditions, of an E. coli-strain used for comparison did not have an embryotoxic effect either.

Hence, according to the investigations, the gramnegative germs, isolated from the different Persian tragacanth specimens, of the genus Enterobacter or their products of metabolism are the cause of the embryotoxic effect of Persian tragacant after intraperitoneal injection for NMRI mice. Nothing precise can be stated about the nature of these products of metabolism of bacteria. Since, however, the used of the autoclave for 15 minutes at 120 C only weakens, but does not eliminates, the ambryotoxic effect of sterile filtrates of broth cultures of Enterobacter, the effective substance must consist at least of a thermolabile and of a thermostable constituents. Whether the thermolabile constituents involves albumins, and the thermostable part possible polysaccharides - like this is known from endotoxins of gram negative germs, must be clarified through further investigations.

Our investigations with decreasing doses of a sterile filtrate of broth culture of enterobacter on the 12th day of gestation show that the number of the dead fetuses decreases in proportion to the dose injected; but that malformations are not increased. Hence it can be concluded that the resorptions and miscarriages, observed in our tests, very probably are no signs of a pure teratogenic action of the products of metabolism of the bacteria.

It is possible that the fetal death is due to massive placenta bleedings, caused by the products of bacteria metabolism of the Enterobacter strains. This might shown by the observation that in some mice, killed immediatly after vaginal bleeding was clinically found, fresh bleedings were detected on the surfaces of the placenta without any fetal residues (state after abortion) and in those with attached fetal sack in which the fetuses in the process of resorption lay. Placenta bleedings, connected with the death of the fetuses, were observed also by THIERSCH (1960) in pregnant rats after injection of lipopolysaccharides, obtained from gram negative bacteria. The cause of the placenta bleedings

could be a disturbance of the blood coagulation, since for dogs, intravenous injections of tragacanth mucilage are deadly on account of the effect exerted on the blood coagulation (WALTON and others, 1959). Such a mechanism could also be implied by the increase of the vascular permeability and lengthening of the time of coagulation found as a result of lipopolysaccharide action, by WESTPHAL and Collaborators (1955).

After repeated subcutaneous injection, a tragacanth mucilage, contaminated with bacteria of the genus Enterobacter, as well as the sterile filtrates of broth cultures manufactured from this tragacanth charge and Enterobacter cloacae 4034, did not influence the fetal development of the NMRI mice. Hence it must be assumed that the embryotoxic effect, observed after intraperitoneal injection in NMRI mice, of different Persian tragacanth charges, which were contaminated with bacteria of the genus enterobacter, and of sterile filtrate of broth cultures of Enterobacter, is not due to a systemic effect, as for instance a general disturbance of the blood coagulation, but to a direct action on the uterus and its vascular system.

For the practice, it results from the investigations presented in this study that:

1. Suspensions and emulsions, which can be contaminated by bacteria of the genus Enterobacter, should at much as possible not be used in testing the possible teratogenic effect of water insoluble substances on the mouse by means of intraperitoneal injection, and

2. Substances, which cause an increasing of the vascular permeability, or possess considerable vasodilatatory or vasoconstrictory effect, and must be applied parenterally, should not be injected intraperitoneally when testing the teratogenic effect, in order to prevent a fetal injury caused by a local perfusion disturbance of the uterus.

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Quantitative Determination of Carrageenan in Milk and Milk Products Using Papain and Cetyl Pyridinium Chloride

SUMMARY—Proteins in milk, chocolate milk, evaporated milk, and ice cream containing added carrageenan were digested with papain at 70°C in the presence of 1.0 *M* NaCl. The digest was adjusted to pH 8.0 to 8.5 with NaOH. Celite was added and the mixture filtered over glass wool. Carrageenan in the filtrate was precipitated with cetyl pyridinium chloride (C.P.C.) in the presence of 0.5 to 1.0 *M* KCl and Celite. The carrageenan-C.P. precipitate was washed with 0.1% C.P.C.-0.05 *M* KCl until the filtrate was negative to the Benedict's test. Then, it was dissolved in 30% H_2SO_4 and the carbohydrate content determined by the phenol-H_2SO_4 method.

At concentrations of 0.01 to 0.2% carrageenan, average recoveries of 92 to 102% were obtained from milk. For chocolate milk, evaporated milk and ice cream, and at a level of 0.1% carrageenan, recoveries of 90, 94 and 96%, respectively, were obtained. Optimum conditions for the isolation of the carrageenan-cetyl pyridinium complex were established.

INTRODUCTION

SEVERAL METHODS ARE AVAILABLE for the isolation, identification and quantitative determination of carrageenan (Ewart *et al.*, 1952; Hansen *et al.*, 1960; Graham *et al.*, 1962; Stoloff *et al.*, 1964; Graham, 1966). However, because of the strong interaction of the hydrocolloid with proteins, the determination of this sulfated polyanionic phytocolloid in foodstuffs containing a high percentage of protein has been tedious.

Proteolytic enzymes such as papain, trypsin, pepsin or pancreatin have been used in the preliminary digestion of proteins in mixtures from which sulfated mucopolysaccharides structurally related to carrageenan were subsequently isolated (Scott, 1960; Korn, 1959a, 1959b; Schiller *et al.*, 1961). In the isolation step, quaternary ammonium detergents have been employed because they can selectively precipitate the sulfated polymers in the presence of salt solutions of fairly high molarity. The insolubility of the carrageenan cetyl pyridinium complex has been mentioned (Scott, 1956, 1960; Slack, 1958).

This paper reports efforts to determine carrageenan in some high protein food products using papain to digest the protein and subsequently determining the carrageenan in the digest after its precipitation with cetyl pyridinium chloride in the presence of relatively high salt concentrations. Various factors which influence the proposed method were investigated and data are given on determination of carrageenan in the presence of other food gums.

EXPERIMENTAL

Materials

A 1% stock dispersion of carrageenan was dialyzed as outlined previously (Graham, 1960).

Buffer: 1.0 *M* sodium acetate-acetic acid, pH 6.5.

Sulfuric acid: (Fisher Scientific Co.) 95 to 98%, sp. gr. 1.84.

Celite 535: (Johns-Manville Co., New York, N. Y. This was washed with distilled water, 1% sodium acetate, sodium chloride (¼ saturated) and finally deionized water until the washings gave a negative phenol-H_2SO_4 test.

Filtration column: The column, 18 mm in diameter and 40 cm high, was made from borosilicate glass and was fitted with a stopcock. It was packed tightly with glass wool to a height of 10 cm. Filtration was done at the rate of 10 to 15 ml per min. The glass wool was washed consecutively with concentrated H_2SO_4, and deionized water until the washings gave a negative phenol-H_2SO_4 test. After this, 25 ml of 0.1% C.P.C.–0.05 *M* KCl was poured over it, with the stopcock closed. The column was soaked in this mixture for at least 30 min and was kept wet with this solution until ready for use.

Papain: This was obtained from the Mann research laboratories as a powdered concentrate assaying 72 milk clotting units per gram by the method of Balls *et al* (1937). Any other highly purified concentrate may be used.

Papain-EDTA-cysteine hydrochloride mixture. Papain (20.0 g), cysteine hydrochloride (1.0 g), and EDTA (1.0 g), were dispersed in 250 ml of warm (70°C) acetate buffer (pH 6.5).

Equipment

Beckman DU-2 spectrophotometer or Bausch and Lomb Spectronic 20 spectrophotometer; pH meter, Beckman Zeromatic.

All glassware was washed thoroughly in water, soaked in dichromate-H_2SO_4 cleaning solution, washed and finally rinsed with distilled water.

Procedure

Digestion of the protein with papain. Five g of milk or milk product was placed in a 250-ml centrifuge bottle and 1.25 to 10.0 mg of carrageenan, dispersed in distilled water, was added. Enough solid sodium chloride to provide a 1.0 *M* solution was added and the total volume made up to 100 ml with distilled water. The mixture was shaken and heated for 10 min in a water bath (98 ± 1°C), cooled to 70°C and 5.0 ml of 1.0 *M* sodium acetate-acetic acid buffer, pH 6.5 was added. The mixture was incubated

at 70°C for 30 min and then 25 ml of the papain-EDTA-cysteine hydrochloride mixture was added. The centrifuge bottle was stoppered with a hard No. 6 rubber stopper, shaken well and the mixture incubated at 70°C for 16 hr. At the end of this period, 2.0 ml of 1.0 *N* NaOH (or enough to adjust the pH of the mixture to 8.5), and 1.0 g of Celite 535 were added. The mixture was shaken well and filtered over glass wool. The residue was washed 3 times with 10-ml portions of distilled water, the washings collected and pooled with the filtrate in a 250-ml centrifuge bottle. The residue was discarded.

Precipitation of the carrageenan. To the filtrate containing the carrageenan, enough solid KCl was added to give a final concentration of 1.0 *M*, then 10 ml of a 1% solution of C.P.C. in water was added drop-wise, with constant shaking. The mixture was shaken well and incubated for 30 min at 45°C. One g of Celite 535 was added, the mixture was shaken and incubated for another 10 min. At the end of this period, the mixture was centrifuged for 10 min at 2,500 r.p.m. and the supernatant filtered over glass wool. The residue containing the carrageenan-C.P. precipitate was retained.

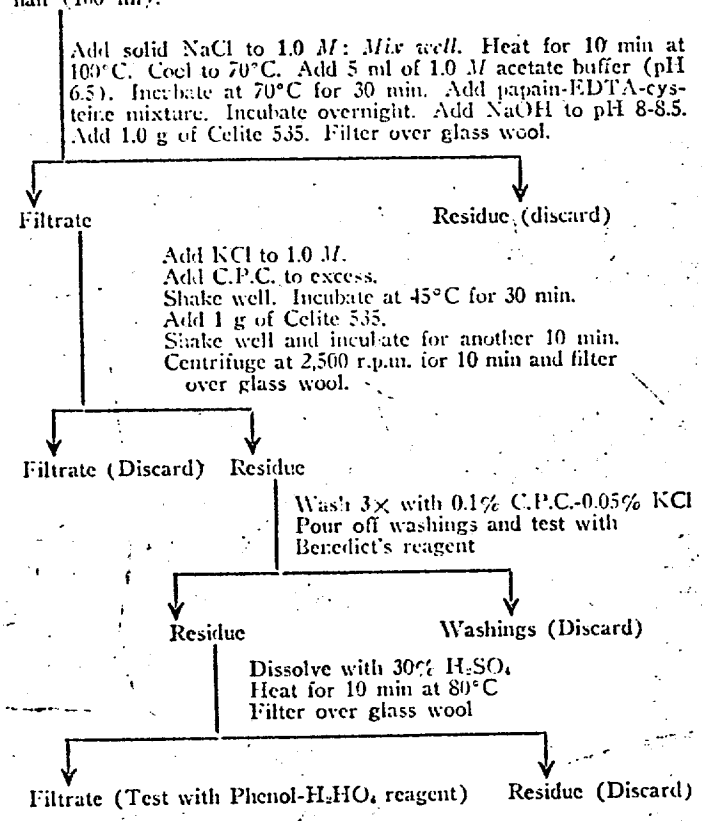
Removal of sugars from the carrageenan-C.P. precipitate. The residue described above contains the carrageenan-C.P. complex adsorbed onto the Celite. Admixed with this are sugars and probably other water-soluble carbohydrates contained in the product. In order to free the precipitate of these interferences, the residue was washed three times with 15-ml portions of 0.1% C.P.C.—0.05% KCl and centrifuged at 2,500 r.p.m. The supernatants were poured over the column of glass wool. The filtrate from the column was tested with the Benedict's reagent and if necessary, the washing was continued until a negative test was obtained. The washings were discarded.

Solution of the carrageenan-C.P. precipitate and determination of the carbohydrate content. To the sugar-free residue 20 ml of 30% H_2SO_4 was added, the precipitate stirred with a glass rod and the mixture held for 10 min in a water bath at 80°C. The mixture was centrifuged for 10 min at 2,500 r.p.m., the supernatant poured over the column of glass wool and the filtrate collected in a 50-ml glass-stoppered volumetric flask. The residue was washed twice more with 12-ml portions of hot (80°C) 30% H_2SO_4, centrifuged, filtered, and the washings also collected. The filtrate and washings was made up to 50 ml with 30% H_2SO_4, mixed well, and a 2-ml portion used for determination of the carbohydrate content by the phenol-H_2SO_4 method. The entire process is summarized in Table 1.

Phenol-H_2SO_4 test. The procedure used, outlined by Dubois *et al.* (1956), is as follows:

Two ml of the carrageenan suspension dispersed in 30% H_2SO_4 and containing 50 to 400 μg of the phytocolloid was placed in a series of 30-ml borosilicate test tubes. One ml of a solution of 5% phenol in water was added and the tubes were shaken to mix the contents well. Five ml of concentrated H_2SO_4 were added rapidly from a burette, allowing the acid to fall directly into the center of the tube in order to obtain good mixing. The tubes were shaken and left at room temperature (28 to 30°C) for 10 min. At the end of this period, the tubes were held in a water bath for 10 min at 28 ± 2°C. The color developed was measured at 490 μ against a reagent blank. This constituted the standard curve.

Table 1. Procedure for separation and determination of carrageenan. 5 g milk (or other product) in 250-ml centrifuge bottle carrageenan (100 ml).



For the determination of carrageenan recovered from milk or other products, 2.0 ml of the filtrate (appropriately diluted, if necessary) were used and the procedure for color development was the same. The blank was treated in the same way as the samples.

Variables affecting the method

Several factors may influence the isolation of carrageenan from any one of the products. These include the time of incubation with the papain, the amount of papain (of known activity) used, the pH of the system, the temperature of incubation, the time of incubation during digestion of the proteins, the completeness of washing of the residue, the pH of the buffered system during digestion of the proteins, the protein content of the sample, the presence of salt in the digestion mixture, the presence of salt during precipitation and the heating of the sample prior to the addition of the papain.

In order to assess the influence of these variables, a constant amount of milk (5.0 g) containing a constant amount of carrageenan (5.0 mg) was subjected to the general procedure while varying the above-mentioned factors one at a time (Table 2).

The optimum conditions for the variables were established by determining the amount of carrageenan recovered. For the first seven variables, the completeness of digestion

Table 2. Influence of variables on the determination of carrageenan in milk

Variable investigated	Range investigated	Condition for reproducibility min.	Condition for reproducibility max.	Value or condition selected
Final concentration of NaCl added (M)	0.0 to 1.5	0.5	1.0	1.0
Time of pre-heating at 99 ± 1°C (min)	0.0 to 30	5.0	10.0	10.0
Final concentration of buffer during incubation (*M*)	0.0 to 0.5	0.1	0.2	0.2
pH of buffered system during digestion	4.0 to 7.0	6.0	6.5	6.5
Temperature of incubation (°C)	40 to 70	65	70	70.0
Time of incubation at 70°C (hr)	1.0 to 36	4.0	16	16.0
Amount of papain added (g)	0.0 to 5.0	1.5	2.0	2.0
Final adjusted pH of digest	6.5 to 10.0	8.0	8.5	8.5
Amount of Celite added before filtering off carrageenan (g)	0.0 to 2.0	0.5	1.0	1.0
Final concentration of KCl added (*M*)	0.0 to 2.0	0.5	1.0	1.0
Amount of C.P.C. added (mg)	1.0 to 100	25	100	100
Amount of Celite added to adsorb precipitate (g)	0.0 to 2.5	0.75	1.0	1.0
Temperature of incubation (°C)	28 to 60	37	45	37.0
Number of washings of precipitate	0.0 to 6.0	Till negative to Benedict's test		Till negative to Benedict's test

was also determined according to the method outlined by Moore *et al.* (1954) and employed by Hill *et al.* (1952).

Recovery of carrageenan

After optimum conditions were established, the recovery of carrageenan from milk, various milk products and from milk to which other gums were added was attempted. The protein content of the various products was determined by the micro-Kjeldahl method as modified by Rao *et al.* (1960). The conversion factor of 6.38 was used to convert nitrogen to protein. Recovery from milk (3.2% protein) was tested by adding varying levels of carrageenan (0 to 10.0 mg) to 5.0 g of milk (Table 3).

Table 3. Recovery of various amounts of carrageenan from milk.

Milk added	Carrageenan added	Carrageenan recovered Range	Carrageenan recovered Average[1]	Recovery (Average)
g	mg	mg	mg	%
5.0	0.00	0.0	0.0	0.0
5.0	10.00	9.6 –10.5	10.2	102.0
5.0	5.00	4.6 – 5.3	4.9	98.0
5.0	1.50	2.2 – 2.6	2.4	96.0
5.0	1.25	1.10– 1.24	1.15	92.0

[1] Average of five different trials.

Recovery from various milk products was tested by adding 5.0 mg of carrageenan to 5.0 g of each product (Table 4).

Recovery of carrageenan from milk in the presence of other hydrocolloids was tested by adding 5.0 mg of carrageenan to 5.0 g of milk (protein content, 3.38%) in the presence of 5.0 mg of each of the hydrocolloids listed in Table 5.

RESULTS AND DISCUSSION

PRELIMINARY HEATING OF THE MIXTURE of milk and carrageenan denatures the milk proteins, thus rendered them more susceptible to digestion by the papain (Scott, 1960). There might be considerable interaction between hydrocolloids like carrageenan and papain, but, in the presence of relatively high concentrations of sodium chloride this does not occur (Scott, 1960).

Experiments in which the amount of added sodium chloride was varied within the limits indicated in Table 1 demonstrated that much better digestion was attained in the presence of at least 0.5 *M* of the salt. The minimum time necessary for digestion of the milk proteins, under the conditions used, was 4 hr. However, under laboratory practices, it is quite convenient to incubate the mixture

Table 4. Recovery of carrageenan from various products.

Product	Amount of product added	Protein content	Carrageenan recovered Range	Carrageenan recovered Average	Recovery (Average)
	g	%	mg	mg	%
Milk (commercial, homogenized, and pasteurized)	5.0	3.38	4.6-5.3	4.9	98.0
Chocolate milk (3% chocolate added to commercial milk)	5.0	3.44	4.2–4.6	4.5	90.0
Evaporated milk (commercial brand)	5.0	7.33	4.5-5.1	4.7	94.0
Ice cream (commercial, vanilla flavor)	5.0	3.88	4.5-5.2	4.8	96.0

[1] Average of five different trials.

Table 5. Recovery of carrageenan from milk in the presence of other gums.

Milk added	Other gum added	Carrageenan recovered: Range	Carrageenan recovered: Average[1]	Recovery (Average)
	mg	mg	mg	%
50	Quince seed (5)[2]	4.4–4.9	4.7	94
50	Sodium carboxymethyl cellulose (5)	4.3–4.8	4.6	92
50	Starch (soluble) (5)	3.5–4.2	3.9	78
50	Pectin (5)	4.4–4.9	4.6	92
50	Locust bean gum (5)	4.5–4.8	4.6	92
50	Sodium alginate (5)	4.4–4.9	4.6	92

[1]Average of three different trials. 5 mg of carrageenan added in each case.
[2]Numbers in parentheses indicate amount in milligrams of other gum added.

overnight (12 to 16 hr). Therefore, an incubation time of 16 hr was used throughout the investigation.

The addition of sodium hydroxide after the incubation aids in the dissociation of any carrageenan-protein complexes present in the mixture. Although the digestion of proteins by papain can occur over a wide pH range, a level of 6.5 was chosen since under this condition and at 70°C the probability of the hydrolysis of carrageenan was greatly minimized. Protein hydrolysis was more rapid at 70°C than at 40°C. It is known that with most substrates, papain exhibits its optimum activity at 60 to 70°C. Buffer strengths between the levels of 0.1 and 0.2 *M* had no measurable influence on the degree of digestion of the milk proteins and, under the experimental conditions and for the papain sample used, less than 1.5 g led to reduced protein hydrolysis.

Celite added at this stage serves to adsorb proteins, protein degradation products and pigments and thus remove them from the bulk of the liquid containing the water-dispersible carrageenan.

Addition of 0.5 to 1.0 g of Celite prior to filtering off the carrageenan in suspension produced a clear filtrate. Use of less than 0.5 g resulted in a slightly colored filtrate due probably, to the presence of protein degradation products and other materials in the digest. This was particularly true with chocolate milk. However, addition of more than 1.0 g of Celite led to reduced recoveries of carrageenan, due undoubtedly to the adsorption of some of the hydrocolloid by the Celite. This possibility has been pointed out by Scott (1960).

Addition of KCl enhances the flocculation of carrageenan. Sodium chloride or other salts would suffice but KCl was chosen because it is known that the kappa fraction of the hydrocolloid is flocculated by this salt, hence the overall precipitation by the C.P.C.-KCl mixture was enhanced. Though concentrations of less than 0.5 *M* will suffice, this minimum is recommended because under these conditions other hydrocolloids such as sodium alginate, pectin and sodium carboxymethylcellulose will not be precipitated by C.P.C. At the maximum concentration of carrageenan used, 100 mg of C.P.C. was enough to provide an excess of the precipitant in the system and 0.75 to 1.0 g of Celite allowed for rapid and complete sedimentation of the precipitate.

Maximum precipitation, as indicated by recoveries using carrageenan in aqueous suspensions, occurred at 37 to 45°C in concurrence with previous observations (Graham *et al.*, 1962). The number of washings of the precipitate necessary in order to remove extraneous carbohydrates differs from product to product. A negative Benedict's test served as a useful end point. The phenol-H_2SO_4 test was used also. However, when large amounts of water-soluble carbohydrates are present, the precipitate must be washed several times. Since this precipitate is not absolutely insoluble in water (Scott, 1960), the phenol-H_2SO_4 test, being extremely sensitive, may at times erroneously indicate incomplete washing. The use of a 0.1% C.P.C.-0.05 *M* KCl solution for washing of the precipitate minimizes this possibility.

Good recoveries of carrageenan from milk were obtained (Tables 3 and 4). Recovery appeared to decrease as the concentration of added carrageenan decreased. Evaporated milk and chocolate milk gave lower recoveries (Table 4). In the latter product, the chocolate on being adsorbed to the Celite added before filtration of the carrageenan in suspension, probably occluded some of the hydrocolloid, thus leading to a lowered recovery.

In the presence of other food gums, the recovery of carrageenan from milk was consistently lower than when the hydrocolloid alone was present. Lowest recovery was obtained in the presence of starch. In all cases, increased viscosity and probably some interaction between the other gums and carrageenan could have subscribed to the results obtained.

Experiments on the recovery of carrageenan from soups gave extremely low results. This might have been due to the large amount of starch in such products. If so, this is in accord with the low recoveries obtained in the presence of starch (Table 5). This difficulty could probably be overcome by pre-digesting the mixture with amylase to degrade the starch. Cursory trials with beer gave average recoveries of 92 to 96% at levels of added carrageenan of 0.02 to 0.2% of the beer.

High results were obtained with the resorcinol, anthrone and Molisch methods due to interference from the C.P.C. and probably traces of proteins (enzymes) in the filtrate. The phenol-H_2SO_4 method was suitable because neither proteins nor C.P.C. interfered. Attempts to further purify the filtrate with charcoal led to low results.

Good recovery of carrageenan from the products used depends greatly on the digestion of the proteins and the removal of degradation products and other colored materials such as those which are present in chocolate milk. After an incubation period of from 4 to 32 hr at 70°C, the mixture of milk and carrageenan gave no increased absorbance at 570 mμ (Moore *et al.*, 1954, Hill *et al.*, 1962), indicating complete hydrolysis of the milk proteins.

As compared to milk, protein digestion in the other products was calculated to be: chocolate milk 91%, evaporated milk 95%, ice cream 96%. In the presence of starch, the digestion of milk proteins was 84%, indicating some retardation of proteolysis which probably was partially contributory to the lowered recovery in the presence of starch.

Trypsin, pepsin and pancreatin, or combinations thereof,

gave much lower recoveries of carrageenan due to poor hydrolysis of the milk proteins.

The proposed method is highly specific because in the presence of 1.0 *M* KCl, other common non-sulfated food gums will not be precipitated by C.P.C. However, its application to individual products should be preceded by a thorough investigation of all variables concerned since the recovery of carrageenan can be influenced by the quantity of one or more ingredients. Possible interference from other sulfated polysaccharides such as furcellaran should not be overlooked.

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Complex Formation Between Hydrocolloids and Tranquilizers and Hypotensive Agents

By HORACE D. GRAHAM, YEDE MARIE BAKER, and A. N. NJOKU-OBI†

Highly insoluble complexes are formed between hydrocolloids such as carrageenan and sodium carboxymethylcellulose and sodium alginate, and tranquilizers and hypotensive agents such as promazine hydrochloride, chlorpromazine hydrochloride, and reserpine. Meprobamate and hexamethonium chloride form much weaker complexes as evidenced by their failure to produce turbidity with several hydrocolloids tested. Using the promazine hydrochloride–carrageenan (Seakem type 5) interaction as the experimental system, it was shown that maximum interaction occurred at 45° and at a pH of 4.95 in a buffered medium. After intramuscular injection of the carrageenan–promazine hydrochloride complex into rabbits, free promazine was detected in the blood up to six days after administration as contrasted to the disappearance of the free form, similarly administered, after only 24 hours.

RECENTLY, chlorpromazine has gained much attention, not only because of its tranquilizing effect but also because of its interaction with sulfated mucopolysaccharides such as chondroitin sulfate and other biochemical compounds (1–4). Since chondroitin sulfate occurs in dentin and enamel and is produced in excessive quantities in abnormal calcification of the arteries and bone (5, 6), such interactions are important pathologically. In one specific study, Sobel and Burger (6) exploited it to study the mechanism of dentin formation. DeLuca and Kostenbauder (7) mentioned that drugs such as chlorpromazine, promethazine, and tetracaine hydrochlorides are bound by nonionic surfactants but, as far as can be ascertained, a comprehensive study of the binding of tranquilizers and hypotensive agents by hydrocolloids has apparently not been made.

Many tranquilizers, when given in large doses, may cause several undesirable side effects. On the other hand, if small compatible doses are given, the treatment must be repeated at frequent intervals. For these reasons, sustained release dosage forms of many medicinals have become of primary importance.

Chemically, sulfated hydrocolloids like carrageenan, furcellaran, fucoidan, etc., are similar to chondroitin sulfate, are readily available, and are very reactive. Therefore, it was decided to study the interactions of these natural compounds with tranquilizers and hypotensive agents. It was considered that if highly insoluble complexes could be obtained, these studies would provide some basis for the use of some of these hydrocolloids in the development of sustained release forms of the drugs. Since the hydrocolloids are natural, readily available, and highly innocuous products, their introduction into biological systems would present no medico-legal problems.

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EXPERIMENTAL

Reagents

Hydrocolloids.—These are listed in Table I. Unless otherwise stated, all dispersions were prepared as described by Graham and Thomas (8). All calculations were made on a dry weight basis.

Tranquilizers and Hypotensive Agents.—Promazine hydrochloride, 0.5% in distilled water; chlorpromazine hydrochloride, 0.5% in distilled water; Serpasil phosphate (reserpine), 0.1% in distilled water; veratrine sulfate, 0.5% in distilled water; Veriloid, 0.1% in dilute acetic acid; and veratrine alkaloid, 0.1% in dilute acetic acid.

Equipment

The following were used: a Coleman Universal spectrophotometer, model 14; a Beckman pH meter, model G; Pyrex glass-stoppered test tubes; and dialysis membrane, 27/32 dialysis tubing, seamless cellulose.[1]

Procedure

General Survey of the Interaction of Hydrocolloids with Tranquilizers and Hypotensive Agents.—In order to obtain some idea of the reaction of a broad spectrum of hydrocolloids with several tranquilizers and hypotensive agents, a screening program was undertaken. For this, 1–5 ml of each hydrocolloid suspension was mixed with 2 ml of the stock solution of each tranquilizer or hypotensive agent and the development or nondevelopment of turbidity noted. The results of this study are summarized in Table I.

Relative Reactivity of Hydrocolloids with Tranquilizers and Hypotensive Agents. Since in some

[1] From Fisher Scientific Co.

cases only slight precipitation was observed in the screening program, more information was sought on the relative reactivity of the hydrocolloids with tranquilizers and hypotensive agents. The sulfated hydrocolloids were previously observed to be precipitated most readily by the drugs in question. Moreover, the turbidity developed was shown to be proportional to the amount of hydrocolloid present. Based on this premise, a scheme was developed for comparing the reactivities of the hydrocolloids.

One to five ml. containing 1–10 mg. of the particular hydrocolloid was mixed with 2 ml. of the stock solution of the particular tranquilizer or hypotensive agent at 45°. The total volume in all cases was 10 ml., attained by adding distilled water where necessary. The reaction was allowed to proceed for 15 minutes, the tubes were cooled to room temperature and the turbidity developed measured at 400 mμ, with a Coleman Universal spectrophotometer, model 14. Since some solutions were appreciably viscous, accompanying control tubes were included by substituting 2 ml. of distilled water for the solution of tranquilizer or hypotensive agent. The actual turbidity (optical density) developed, therefore, was calculated as "the turbidity measured minus the turbidity (if any) of the accompanying control."

In order to compare the reactivities of the hydrocolloids, the turbidity produced by 1 ml. of carrageenan (Seakem type 5) with a particular tranquilizer or hypotensive agent was assigned an arbitrary value of 10. The turbidity produced by 1 mg. of any other hydrocolloid through interaction with the same tranquilizer or hypotensive agent was then compared with the value obtained for carrageenan (Seakem type 5) to give values recorded in Table II.

Influence of Variables on the Interaction.—Several variables such as temperature, pH, buffer normality, interaction time, and the presence of inorganic salts could possibly influence the binding of the drugs by the hydrocolloids. Therefore, these variables were systematically investigated. Unless otherwise stated, the carrageenan (Seakem type 5)-promazine hydrochloride system was used throughout the investigations on variables.

The influence of temperature was assessed by reacting 10 mg. of the hydrocolloid with 2 ml. of the drug solution at 30, 45, and 65° in a total volume of 10 ml. (Where reactions were done at 45 and 65°, the tubes were immediately cooled in an ice water bath after the desired reaction time.) After adjusting to room temperature, the turbidity (optical density) developed was measured at 400 mμ with the Coleman Universal spectrophotometer, model 14. For the establishment of the optimum

TABLE I.—SURVEY OF THE INTERACTION OF HYDROCOLLOIDS WITH TRANQUILIZERS AND HYPOTENSIVE AGENTS[a]

Hydrocolloids	Tranquilizers: Promazine Hydrochloride	Tranquilizers: Chlorpromazine Hydrochloride	Hypotensive Agents: Reserpine	Hypotensive Agents: Veriloid	Hypotensive Agents: Veratrine Alkaloid	Hypotensive Agents: Veratrine Sulfate
Group I.—Sulfated polysaccharides						
Carrageenans (Seakem types 4, 5, 7, 402)	+	+	+	+	+	+
Furcellaran	+	+	+	+	+	+
Sodium kappa carrageenan	+	+	+	+	+	+
Sodium lambda carrageenan	+	+	+	+	+	+
Gigartina acicularis	+	+	+	+	+	+
Gigartina pistillata	+	+	+	+	+	+
Eucheuma cottonii	+	+	+	+	+	+
Eucheuma spinosum	+	+	+	+	+	+
Fucoidan	+	+	+	+	+	+
Agar	+	±	−	−	±	+
Hypnean	+	+	+	+	+	+
Iridophycan	+	+	+	+	±	+
Gelcarin	+	+	+	+	+	+
Group II.—Carboxylic acid type polysaccharides						
Gum karaya	+	+	−	−	±	−
Gum arabic	−	−	−	−	±	±
Gum tragacanth	−	−	−	−	±	+
Gum ghatti	−	−	−	−	+	±
Pectin	+	+	−	−	±	+
Sodium carboxymethylcellulose	+	+	+	+	±	+
Sodium alginate	+	+	+	+	±	+
Group III.—Neutral polysaccharides						
Locust bean gum	−	−	−	−	−	−
Gum guar	−	−	−	−	±	−
Potato starch	−	−	−	−	−	−
Group IV.—Proteins						
Casein	+	+	−	−	+	+
Gelatin	−	−	−	−	±	±
Group V.—Other compounds						
Heparin	+	+	+	+	+	+
Chondroitin sulfate	+	+	+	+	+	+
Polyglucose sulfate	+	+	+	+	+	+

[a] + = precipitation; − = no precipitation; and ± = gelation.

TABLE II.—RELATIVE REACTIVITY OF THE HYDROCOLLOIDS WITH TRANQUILIZERS AND HYPOTENSIVE AGENTS

Hydrocolloids	Tranquilizers: Promazine Hydrochloride	Tranquilizers: Chlorpromazine Hydrochloride	Hypotensive Agents: Reserpine	Hypotensive Agents: Veriloid	Hypotensive Agents: Veratrine Sulfate
Group I.—Sulfated polysaccharides					
Carrageenan Seakem type 5	10.00	10.00	10.00	10.00	10.00
Carrageenan Seakem type 402	10.62	11.20	9.85	14.00	11.40
Furcellaran	5.39	5.80	6.70	5.4	6.2
Sodium kappa carrageenan	10.55	10.20	9.98	10.6	10.8
Sodium lambda carrageenan	16.00	15.80	14.79	13.2	14.6
Viscarin	9.74	10.4	11.94	10.8	11.97
Agar	0.08	0.11	0.46	0.08	0.40
Gelcarin	12.95	11.20	9.98	11.2	9.92
Group II.—Carboxylic acid type polysaccharides					
Gum karaya	1.03	0.98	0.06	0.04	0.26
Gum arabic	0.03	0.01	0.09	0.01	0.04
Gum tragacanth	0.00	0.09	0.00	0.00	0.00
Gum ghatti	0.09	0.08	0.16	0.01	0.01
Pectin	0.04	0.04	0.15	0.04	0.04
Sodium carboxymethylcellulose	0.84	0.82	6.18	2.76	0.06
Sodium alginate	0.48	0.47	0.70	0.82	0.36
Group III—Neutral polysaccharides					
Locust bean gum	0.01	0.04	0.71	...	0.03
Gum guar	0.34	0.05	0.33	...	0.60

interaction time at 45°, mixtures of the hydrocolloid and the drug in a total volume of 10 ml. were allowed to interact for the periods shown in Table III and the optical density measured as described above. Studies on the effects of pH were done by adding 2 ml. of 0.1 *M* phosphate buffers of varying pH levels to test tubes containing a suspension of 10 ml. of the hydrocolloid and 2 ml. of the drug solution. The total volume was always 10 ml. The mixtures were incubated at 45° for 15 minutes and, after cooling to room temperature, the turbidity that developed was measured at 400 mμ. The final pH of each system was checked with a Beckman model G pH meter. Since the drugs gave precipitates with buffer salts in the alkaline range, all experimentation was restricted to the acid range. Any influence of buffer molarity was ascertained by adding varying amounts of 1.0 *M* phosphate buffer, pH 4.95, to test tubes containing 1 ml. of the hydrocolloid suspension and 2 ml. of the drug solution. After making the volume 10 ml., the turbidity developed at 45° for 15 minutes was measured as described above.

Since pharmaceutical formulations may contain certain salts, the influence of some of the more common salts on the intensity of the interaction was

TABLE III.—INFLUENCE OF VARIABLES ON THE INTERACTION OF CARRAGEENAN[a] WITH PROMAZINE HYDROCHLORIDE

Variable	Absorbance at 400 mμ: Immediately Measured	Absorbance at 400 mμ: After Cooling and Brought to Room Temperature
Temp., (° C.)		
30	0.153	0.155
45	0.220	0.324
65	0.142	0.318
Reaction Time, Min. at 45° C.		
0	0.180	0.300
5	0.200	0.320
10	0.205	0.320
15	0.215	0.320
30	0.215	0.325
36	0.210	0.370
45	0.195	0.370
90	0.240	0.330
Buffer Molarity		
0.01	...	0.198
0.05	...	0.198
0.10	...	0.200
0.30	...	0.185
0.50	...	0.181
0.70	...	0.145
pH	Promazine Hydrochloride Bound μM/mg. of Carrageenan (Seakem type 5)	
5.50 (unbuffered)	...	1.40
2.55	...	1.15
2.85	...	1.18
4.45	...	1.20
4.95	...	1.55

[a] Seakem type 5.

TABLE IV.—INFLUENCE OF ADDED SALTS ON THE INTERACTION OF CARRAGEENAN[a] WITH PROMAZINE HYDROCHLORIDE AT 45° C.

Salt Added as Chloride	Tolerance Level, *M*
Monovalent	
Li^+	4.25×10^{-4}
K^+	3.0×10^{-4}
NH_4^+	6.4×10^{-4}
Na^+	5.0×10^{-4}
Divalent	
Mg^{++}	2.5×10^{-4}
Ba^{++}	5.0×10^{-4}
Cd^{++}	1.1×10^{-4}
Zn^{++}	1.4×10^{-4}
Sr^{++}	1.4×10^{-4}
Mn^{++}	2.5×10^{-4}
Ca^{++}	1.0×10^{-4}
Trivalent	
Al^{+++}	1.2×10^{-4}
Fe^{+++}	1.0×10^{-4}

[a] Seakem type 5.

assessed. This was done by adding varying amounts of each salt solution to the reaction tubes and measuring the optical density as described above. Duplicate control tubes containing no added salt were included in each experiment. The tolerance level of each salt was established in a manner similar to that employed by Graham and Thomas (8). It was taken as "That maximum final concentration (the concentration of salt in the 10 ml. of reaction medium) of salt which did not cause a significant difference (in this case ±1%) in the absorbance as compared to a control tube to which no salt was added." The results are summarized in Table IV.

Maximum Binding Capacity of Hydrocolloids for the Tranquilizers and Hypotensive Agents.—More detailed quantitative data on the binding process were obtained by determining the maximum binding capacity of representative hydrocolloids for promazine hydrochloride and reserpine. For this, 5 ml. of the suspension containing 50 mg. of the particular hydrocolloid was allowed to react at 45° for 15 minutes with increasing quantities of the tranquilizer or hypotensive agent, in a centrifuge tube, and with a total volume of 10 ml. After cooling to room temperature, the reaction mixture was centrifuged at 1500 r.p.m. for 10 minutes. One milliliter of the supernatant was placed in 9 ml. of methyl alcohol and this alcohol mixture again centrifuged as above. One milliliter of this supernatant was then used for the colorimetric determination of the particular tranquilizer or hypotensive agent. The amount of the drug bound was calculated as "the amount added minus the amount found free in the supernatant."

Colorimetric Methods for Determination of Tranquilizers and Hypotensive Agents. Promazine was determined according to the method of Leach and Crimmin (9). Reserpine was determined by the method of Indemans, *et al.* (10).

Calculation of the Maximum Binding Capacity.—The maximum amount of promazine hydrochloride and reserpine bound per gram of three representative hydrocolloids was established from a Langmuir type plot as employed by Kennon and Higuchi (11) and Patel and Kostenbauder (12). By plotting the reciprocal of the amount of drug bound per unit of hydrocolloid on the *Y* axis *vs.* the reciprocal of the amount of free drug in the system, and extrapolating to zero concentration of the drug, a line which cuts the *Y* axis was obtained. The *Y* intercept gives a measure of the maximum binding capacity (13). The results are summarized in Table V and representative graphs are shown in Figure 1.

Solubility of the Promazine Hydrochloride–Hydrocolloid Complexes.—Since the promazine hydrochloride–carrageenan complex was so readily formed and seemed highly insoluble, it was deemed useful to study its solubility in solutions of sodium chloride and of hydrochloric acid. The method used was similar to that employed by Antonopoulous, *et al.* (14), in studying detergent complexes and Bridger, *et al.* (15), in studying metal ammonium phosphate complexes. For this, 5 ml. of a dispersion containing 50 mg. of carrageenan (Seakem type 5) and 5 ml. of a 0.5% solution of promazine hydrochloride were allowed to react at 45° for 30 minutes in 15-ml. graduated centrifuge tubes. After cooling, the mixture was centrifuged at 2500 r.p.m. for 10 minutes and the supernatant carefully poured off. The precipitate was washed five times with distilled water and centrifuged after each washing. The precipitate from the centrifuge tubes was added to consecutive 200 × 15 Kimax test tubes and quantities of 4 *M* sodium chloride or 2 *N* hydrogen chloride added to give the final normalities shown in Table VI. The final volume was 20 ml. in all cases attained by adding distilled water where necessary. The tubes were shaken on a Burrell wrist action shaker at 27° and, at the intervals shown, 3 ml. of the

TABLE V.—MAXIMUM BINDING CAPACITY OF HYDROCOLLOIDS FOR PROMAZINE HYDROCHLORIDE AND RESERPINE AT 45° C.

Hydrocolloids	μM of Drug Bound/Gm. of Hydrocolloid: Promazine Hydrochloride	Reserpine
Carrageenan (Seakem type 5)	1500	666.0
Na carboxymethylcellulose	244	540.5
Pectin	14.3	238.0

TABLE VI.—SOLUBILITY OF THE PROMAZINE HYDROCHLORIDE-CARRAGEENAN[a] COMPLEX IN SODIUM CHLORIDE AND HYDROCHLORIC ACID AT 27° C.

	% of Bound Promazine Released After: 1 Hr.	6 Hr.	12 Hr.	24 Hr.
	Sodium Chloride (Final Molarity)			
0.0 (H_2O)	4.000	4.01	4.17	4.87
0.2	26.52	29.46	30.55	29.17
0.4	29.65	31.63	34.72	38.88
1.6	38.74	40.42	48.61	43.75
	Hydrochloric Acid (Final Normality)			
0.0 (H_2O)	4.00	4.01	4.17	4.87
0.01	7.00	7.82	8.33	13.18
0.1	18.46	20.01	23.61	30.00
0.2	25.20	26.52	30.55	36.11
0.5	24.62	29.80	38.19	58.33
1.0	40.80	48.60	57.08	65.29
2.0	56.40	60.00	79.17	83.33

[a] Seakem type 5.

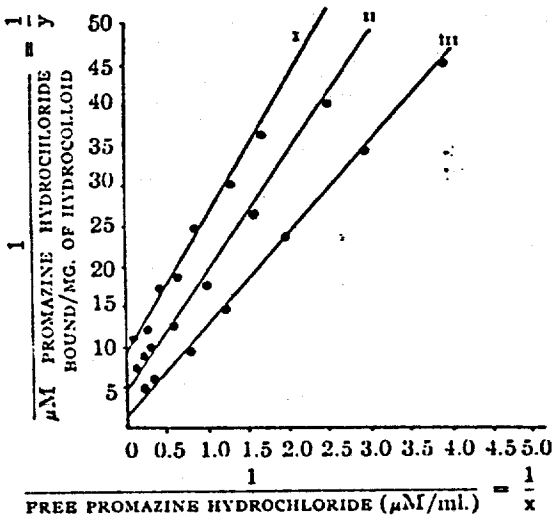

Fig. 1.—Langmuir type plot of data to determine maximum binding capacity of hydrocolloids for promazine hydrochloride. I = pectin; II = sodium carboxymethylcellulose; and III = carrageenan (Seakem type 5).

mixture centrifuged and one ml. of the supernatant assayed for promazine released from the complex. The results are shown in Tables VI and VII. For comparison, the promazine hydrochloride-sodium carboxymethylcellulose complex was also studied.

Experiments on the Promazine Hydrochloride–Carrageenan Complex.—Batch quantities were prepared by using a 200-ml. suspension containing 200 mg. of carrageenan (Seakem type 5), preincubated at 45° for 30 minutes. One hundred milliliters of a solution containing 2 Gm. of promazine hydrochloride was similarly preincubated. At the end of the preincubation period, both the hydrocolloid suspension and the promazine hydrochloride solution were mixed in a 1000-ml. Erlenmeyer flask and allowed to interact for 30 minutes at 45°. The mixture was placed in a cold room at 4° overnight and filtered. The filtrate was collected and carrageenan added to precipitate any promazine in it. If precipitation occurred, the cooling and filtration processes were repeated. The precipitates were pooled and washed with distilled water to remove any unreacted hydrocolloid. This was then followed by washing 5 times with absolute methyl alcohol. The precipitate was then collected, placed in a Petri dish and dried overnight in a vacuum oven at 75°. After this, the mass was pulverized in a mortar and stored in a vial.

Titration of the Complex.—In order to determine the promazine content of the complex 10 mg. was placed in a 25-ml. volumetric flask and enough concentrated sulfuric acid added to dissolve it. Water was added to make it up to volume. From this, 1 ml. was taken, further appropriately diluted with distilled water and the promazine content determined colorimetrically by the method of Leach and Crimmin (9).

Sustained Release of the Promazine from the Complex *in vivo*.—In order to ascertain if sustained release of the promazine from the carrageenan (Seakem type 5)–promazine complex could be achieved, free promazine, and the suspending medium, respectively, was injected intramuscularly into individual rabbits. Sterile physiological saline and a 1:5:4 mixture of ethyl alcohol, glycerin, and water were used as suspending media in the separate experiments. Samples of blood were drawn at intervals from 4 hours to 6 days and analyzed for promazine by the method of Leach and Crimmin (9). Simultaneously, changes in the picture of the white blood cells were followed. The results are summarized in Table VIII.

TABLE VII.—SOLUBILITY OF PROMAZINE HYDROCHLORIDE-SODIUM CARBOXYMETHYLCELLULOSE COMPLEX IN SODIUM CHLORIDE AND HYDROCHLORIC ACID AT 27° C.

	% of Bound Promazine Released After			
	1 Hr.	6 Hr.	12 Hr.	24 Hr.
Sodium Chloride (Final Molarity)				
0.0 (H_2O)	10.6	11.4	12.6	14.3
0.2	68.0	71.4	78.0	88.4
0.4	72.0	78.0	87.0	94.3
0.8	75.2	81.6	90.0	98.0
1.6	80.2	87.6	91.2	98.4
2.0	81.6	90.2	94.3	98.7
Hydrochloric Acid (Final Normality)				
0.0 (H_2O)	10.6	11.4	12.6	14.3
0.01	20.6	25.0	28.6	30.2
0.10	28.0	38.0	40.8	53.0
0.20	40.8	46.0	65.8	76.5
0.50	68.7	85.6	93.0	94.6
1.00	72.4	86.2	94.5	96.2
2.00	76.0	87.0	94.3	96.4

RESULTS AND DISCUSSION

From Tables I and II it is clear that the sulfated polysaccharides are the most reactive hydrocolloids. The low reactivity of agar may be partly explained by the fact that its sulfation is still not well established (16, 17). The carboxylic acid type polysaccharides are next in order of reactivity. Of these, sodium carboxymethylcellulose is one of the more reactive. In the experiments with sodium carboxymethylcellulose, all reagents were dissolved in distilled water to avoid precipitation of the hydrocolloid itself at low pH levels of the medium (17). The neutral polysaccharides were the least reactive.

Of the several variables which affect the interaction of promazine hydrochloride with carrageenan (Seakem type 5), the pH of the interaction medium is the most important. In the alkaline range, the tranquilizer will precipitate in the absence of the hydrocolloid, hence only the acid range was investigated. Maximum interaction occurs at pH 4.95, and as the pH decreases the amount of promazine bound per mg. of the hydrocolloid decreases. At a final pH of 2.25, binding of the drug by the hydrocolloid was still appreciable since ionization of the sulfate group is not drastically impeded even at this low final pH. A similar pattern would be expected for the other sulfated hydrocolloids. However, the carboxylic acid type hydrocolloids would be more severely influenced since at pH levels of 2.5–3.0, sodium carboxymethylcellulose, in particular, would precipitate in the absence of the tranquilizer.

The temperature has a great influence on the interaction as exemplified by the promazine hydrochloride–carrageenan (Seakem type 5) interaction. In the acid range (pH 4.95) turbidity is a good measure of the intensity of the interaction and it was used to assess this influence. At 30°, the turbidity developed was lower than at 45° or at 65°. Apparently, at this temperature (30°) ionization of both interactants may not be complete. The great sensitivity of the system to temperature as indicated

TABLE VIII.—DETECTION OF PROMAZINE IN BLOOD OF RABBIT AFTER INTRAMUSCULAR INJECTION OF PROMAZINE HYDROCHLORIDE AND THE CARRAGEENAN-PROMAZINE COMPLEX

	Promazine Detected in Blood After Injection of Carrageenan[a]	
Time Lapse After Injection, Hr.	Promazine Complex μg./ml. of Blood	Promazine Hydrochloride μg./ml. of Blood
0.0	0.0	0.0
4.0	2.0	2.64
12.0	3.07	4.5
18.0	...	1.9
24.0	3.55	0.0
48.0	3.47	...
72.0	1.29	...
96.0	0.95	...
144.0	0.47	...

[a] Seakem type 5.

TABLE IX.—EFFECT OF THE INTRAMUSCULAR INJECTION OF THE CARRAGEENAN[a]-PROMAZINE COMPLEX ON THE PICTURE OF THE WHITE BLOOD CELLS OF THE RABBIT

Time After Injection, Hr.	Variations in Count of White Blood Cells				
	Neutrophils, %	Monocytes, %	Basophils, %	Eosinophils, %	Lymphocytes, %
0.0	26.8	12.0	0	5.0	54.0
4.0	38.0	12.5	0	3	46.0
12.0	19.0	34	0	0	46.0
18.0	...	...	...	...	...
24.0	45.0	29.5	1.0	2	22.5
48.0	29.0	30.5	0	0	20.5
72.0	30.0	32.0	2	0	36.0
96.0	41.0	19.0	2	3	36
144.0	...	...	...	...	...

[a] Seakem type 5.

by the measurements made immediately (before cooling) and those made after cooling to room temperature indicate that, apparently, forces other than the ionic type can participate in the binding process. Since the turbidity developed at 45° was essentially the same as that at 65° (after cooling), 45° was chosen for all later experiments.

Under the experimental conditions employed, at 45° the maximum interaction occurred after 30 minutes. Despite this, a heating time of 15 minutes was selected to avoid any possibility of hydrolysis of the hydrocolloid due to prolonged heating, and to facilitate rapidity of experimentation when many samples were involved. The degree of interaction at 45° for 15 minutes remained constant for as long as 4 hours, hence comparisons based on the time–temperature selection are not invalid.

Final buffer molarities of 0.01–0.10 resulted in no drastic change in the interaction as evidenced by the turbidity measurements. Therefore, where buffers were used, a final molarity of 0.01 was selected.

Table IV summarizes the influence of salts on the promazine hydrochloride–carrageenan (Seakem type 5) interaction. On a molar basis, the order of tolerance of the added salts was found to be monovalent > divalent > trivalent. Severe interference of the salts is due to competition for the binding sites of the hydrocolloids. The trivalent cations will actually precipitate carrageenan and other hydrocolloids, even at relatively low concentrations.

Although only the promazine hydrochloride–carrageenan (Seakem type 5) system was investigated in detail, it is to be expected that the other systems would be similarly affected by the variables listed. As noted with the precipitation with alkaloids (8), lambda carrageenan was always more reactive than kappa carrageenan. This is in line with chemical data which show that the lambda fraction is much richer in ester sulfate content than the kappa fraction (17).

The solubility data recorded in Tables VI and VII indicate that the promazine hydrochloride complexes of carrageenan (Seakem type 5) and sodium carboxymethylcellulose are highly insoluble in sodium chloride and hydrochloric acid. Relatively, the sodium carboxymethylcellulose complex is more soluble than the carrageenan complex in both sodium chloride and hydrochloric acid. This insolubility renders them potentially suitable as sustained release forms of this and possibly other drugs. The need for complexing agents for the development of such depot forms of drugs has recently been clearly emphasized by Hirscher and Miller (18).

Analysis of the data recorded in Table VIII indicate that the promazine hydrochloride–carrageenan (Seakem type 5) complex can serve as a depot form of the drug. The prolonged presence of the drug in the blood of the rabbit leads to the conclusion that the drug was released from the complex. Contrastingly, the disappearance of drug administered as the "free form," after 24 hours, indicates the further superiority of the complex where sustained release is desired. Le Blanc (19) reported that a single injection of chlorpromazine caused a drop in the eosinophils, leucocytes, and platelets in the blood of male albino rats. This effect, in their opinion, was not a direct result of hypothermia. Korst (20) has reviewed several cases of agranulocytosis caused by phenothiazine derivatives including chlorpromazine and promazine. It was noted that in some cases the neutrophils decreased drastically or even vanished and that the total leucocyte count severely decreased. Since promazine hydrochloride is very closely related to chlorpromazine hydrochloride, it was decided to investigate the blood picture changes, if any. The variations in the blood cell picture shown in Table IX, may be considered as further evidence that the promazine was being released from its hydrocolloid complex. Besides the possibility of their use for development of sustained release forms of drugs, the effect of these hydrocolloids on the availability of drugs and other chemicals in pharmaceutical preparations must also be emphasized. It has been shown that gums (hydrocolloids) will lower the availability of tetracycline antibiotics.

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Antipeptic and antithrombic properties of carrageenin

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Carrageenin is a sulfated galactan obtained by water extraction from certain seaweeds, usually Chondrus crispus. It can be fractionated into κ-carrageenin and λ-carrageenin. λ-Carrageenin has a higher sulfate content. Tests in vitro demonstrated that carrageenin inhibited the digestion of egg albumin by purified pepsin and was antithrombic in both human and dog blood plasma. In both antipeptic and antithrombic activity λ-carrageenin was more active than the κ-fraction. An assay invitro indicated that λ-carrageenin, unfractionated carrageenin, and κ-carrageenin have respectively about 1/13, 1/24, and 1/34 the antithrombic potency of heparin. It is pointed out that the anticoagulant property of carrageenin is a contraindication to its use in the treatment of peptic ulcer.

Carrageenin is the common name applied to the polysaccharide that can be extracted with water from species of the red seaweeds Chondrus and Gigartina. It has been separated into two components designated κ- and λ-carrageenins.[1, 2] They are galactans, the former made up of D-galactopyranosyl-3,6-anhydro-D-galactose-4-sulfate[2-5] and the latter mainly, if not entirely, of D-galactopyranose-4-sulfate.[6] Lambda carrageenin has the higher sulfate content.[1]

Sulfated polysaccharides generally may be expected to possess some of the biochemical characteristics of heparin, such as anticoagulant, antilipemic, and antipeptic activities, and these properties have been established for some of them, both natural and synthetic.

The power to inhibit or prolong the coagulation of mammalian blood has been shown for sulfated sodium alginate,[7] sulfated laminarin,[8] dextran sulfate,[9] lactin,[10] fucoidin,[11, 12] and the polysaccharide from *Irideae laminarioides*.[13, 14] Anticoagulant activity in agar is uncertain.[13, 14] The few observations that have been made on carrageenin have indicated that it has slight anticoagulant activity[12, 14] or none.[13] As with heparin, the mode of action of these compounds,

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where it has been studied, is antithrombic.[8, 9, 11, 12] The antilipemic activity of some of these substances has also been established.[16-19]

The antipeptic activity of mucoitinsulfuric acid[20] and of chondroitinsulfuric acid[21] was described almost 30 years ago. More recently it has been established for heparin and sulfated polyanhydromannuronate.[22] In 1959 Anderson and Watt[23] reported that carrageenin inhibited peptic activity both in vitro and in vivo, and prevented the formation of gastric ulcers in guinea pigs. The effect in vitro has been confirmed, and compared with that of heparin by Piper and Fenton.[24] These authors discussed the treatment of peptic ulcer with carrageenin.

In 1957 Houck, Morris, and Lazaro[15] reported experiments on the intravenous injection of algal polysaccharides into dogs. Only that from *Gigartina acicularis* showed anticoagulant activity according to their test. A commercial carrageenin after some purification was inactive at a dosage of 1 mg. per kilogram of body weight.

The experiments described below were undertaken to clarify the position of the carrageenins as anticoagulants and antipeptic agents, principally because of their possible use in the treatment of peptic ulcer in humans. The anticoagulant activity is obviously of importance because of the frequency of hemorrhage in this condition. This work led to an estimation of the potency of κ- and λ-carrageenins as antithrombic and antipeptic agents.

Materials and methods

Tests of blood clotting efficiency: The same materials and methods were used as those previously described in an investigation of laminarin sulfate.[8] Recalcification-thrombin time was determined by substituting $CaCl_2$ solution for the 0.1 ml. of saline in the measurement of thrombin time.

Table I. Antipeptic activity in vitro of carrageenin and of its κ- and λ-fractions*

Amount of test material (mg.)	*Inhibition of digestion (%) Carrageenin*		
	Whole	κ	λ
2		18	
1.5		11	
1	98	9	
0.8	95		14
0.6			10
0.5		0	
0.4	46		
0.2	14		7
0.1	0		6

*Egg albumin was the substrate, in 2.5 per cent stock solution. Pepsin, three times crystallized (General Biochemicals, 1959E), was used in a concentration of 5 mg. per 100 ml. The digestion mixture contained 4 ml. of working solution (2 per cent) of substrate, 1 ml. of enzyme solution, and 1 ml. of H_2O. Digestion was carried out at 25° C. for 20 minutes. In this system the ratio of substrate to enzyme was 400:1. The material to be tested for antipeptic activity was added in the 1 ml. of H_2O in the amount shown. The unfractionated carrageenin was a purified commercial product. The κ- and λ-fractions were made from carrageenin prepared from freshly gathered [illegible]. For comparison: $HgCl_2$, 0.5 mg., gave 8 per cent inhibition; pectin, [illegible] mg., gave 4 and 3 per cent inhibition; soluble starch, 5 mg., gave no inhibition.

Table II. Effect in vivo of heparin upon the clotting efficiency of the blood of the dog*

Dose (mg./Kg.)	*Time after injection (hours)*	*Clotting time (minutes)*	*Thrombin time (seconds)*	*Recalcified-thrombin time (seconds)*
0.3	0	10	15	8
	½	13	27	10
	1	10	19	9
	1½	9	16	9
	2	9	15	8
0.5	0	9	12	7
	½	30	> 30	27
	1	13	26	10
	1½	11	16	8
	2	9	12	7
1.0	0	10	11	6
	½	> 30	> 30	> 30
	1	30	> 30	18
	2	12	13	6

*The figures are averages from 15 experiments on 5 dogs. Changes in recalcification times were roughly parallel to those in the clotting times. There were negligible changes in prothrombin times.

Times are expressed to the nearest minute or second, whichever unit is used, because it is convenient and the discrepancies involved are insignificant. In all cases where > 30 is used to express a time, there was no evidence of a clot for ½ to 1 hour after 30 minutes or 30 seconds.

Test for antipeptic activity. The method was that of Anson,[25] with egg albumin as substrate. Conditions were chosen that gave a degree of digestion that could conveniently be measured within the confines of a straight-line relationship between tyrosine production and enzyme concentrations.

Sulfated polysaccharides. In some experiments a commercial preparation of carrageenin was used (Seaplant Chemical Corporation). It was purified by solution in hot water and precipitation with ethanol. In other experiments carrageenin prepared in this laboratory from freshly gathered *Chondrus crispus* was used, some of which was separated into κ- and λ-fractions by the method of Smith, O'Neill, and Perlin.[3]

The heparin used was the U.S.P. sodium salt (Connaught) with a potency of 110 I.U. per milligram.

Intravenous injection into dogs. These experiments were always done in the morning, when the dogs were fasting. They were anesthetized by the intravenous injection of sodium pentobarbital (about 30 mg. per kilogram) before the anticoagulant was administered, to facilitate sampling of the blood and to relieve the dogs of discomfort. The anticoagulants were dissolved in warm saline, and administered within 2 hours of preparation of the solution. The injections were given through polyethylene tubing into a saphenous vein over periods of 10 to 20 minutes. Withdrawal of blood samples, from the radial and saphenous veins, was timed from the start of injection.

Anticoagulant activity in vitro. The dogs that supplied blood for the assessment of antithrombic activity had not received injections of anticoagulants.

Human blood was drawn from a vein directly into a syringe containing heparin or carrageenin, to measure the clotting efficiency. Freshly drawn blood from the same subject was also mixed with varying amounts of heparin or carrageenin, and the delay in clotting time noted. These experiments were done principally to demonstrate that carrageenin is an [illegible]

coagulant in human blood. On the blood from another human subject an experiment was done to assess the antithrombic activity of the carrageenins.

Results

The results of the experiment on peptic digestion are shown in Table I. They indicate an antipeptic activity for carrageenin. They demonstrate that both κ- and λ-carrageenin are antipeptic, and that the λ-fraction is the more active. In this experiment the two fractions cannot justifiably be compared with the unfractionated carrageenin because they were not prepared from it.

Tables II and III show the effect upon the blood clotting efficiency of the intravenous administration of heparin and of carrageenin to dogs. The results indicate that the minimum anticoagulant dose of carrageenin by this route is about 3 mg. per kilogram of body weight, that with unfractionated carrageenin and with the κ-fraction larger doses do not proportionately affect the whole-blood clotting time, that λ-carrageenin is the most active of the three, and that the effect is principally upon the thrombin time. From the effect upon the thrombin time in vivo, λ-carrageenin and the unfractionated material appear to be, respectively, about 1/10 and 1/40 as potent as heparin.

After the intravenous injection of carrageenin in most cases there was transitory dyspnea, sometimes severe, that began during the injection or soon

Table III. Effect in vivo of carrageenin and of its κ- and λ-fractions upon the clotting efficiency of the blood of the dog*

Dose (mg./Kg.)	Time after injection (hours)	Clotting time (minutes)			Thrombin time (seconds)			Recalcified-thrombin time (seconds)		
		Whole	κ	λ	Whole	κ	λ	Whole	κ	λ
3	0	10	9	9	11	12	14	8	8	8
	½	18	12	13	15	14	18	8	9	9
	1	17	12	10	17	14	19	8	9	8
	2		10	13		15	14		8	9
5	0	9		9	12		13	7		7
	½	13		>30	14		>30	8		13
	1	9		17	13		>30	7		10
	2			15			20			9
10	0	8	10		11	15		7	7	
	½	12	11		16	18		10	9	
	1	10	10		14	18		9	8	
	1½	9	9		14	18		9	8	
20	0	10			13			7		
	½	13			30			16		
	1	11			28			16		

*The figures are averages from [illegible] experiments on 5 dogs. Purified commercial carrageenin was used [illegible] carrageenin prepared in the laboratory from *Chondrus* [illegible] were essentially the same in their effects. Changes in recalcification times were [illegible] parallel to those in the clotting times. There [illegible] in prothrombin times.

Table IV. Comparison with heparin of the anticoagulant effect in vitro of carrageenin in human blood*

Material added	*Clotting time (minutes)*	*Prothrombin time (seconds)*	*Thrombin time (seconds)*	*Recalcified-thrombin time (seconds)*
Measures of clotting efficacy:				
Saline only	9	15	15	7
Heparin (0.1 mg.)	16	20	> 30	> 30
Carrageenin (0.5 mg.)	15	34	> 30	> 30
Delay of clotting‡				
Heparin (mg.)	0	0.005	0.007	0.01
½ hour	+	–	–	–
1 hour	+	+	±	–
Carrageenin (mg.)	0	0.055	0.06	0.065
½ hour	+	–	–	–
1 hour	+	+	±	–

*The carrageenin was a purified commercial product.

†Material added was contained in 1 ml. of saline in a syringe, and 9 ml. of blood was drawn into it from a vein.

‡Anticoagulant was contained in 0.1 ml. of saline in a tube 1 cm. by 7.5 cm. To each was added directly from the syringe 1 ml. of freshly drawn blood, and the contents mixed by one inversion. The mixtures were examined at ½ hour and 1 hour, and the consistency recorded as plus, clotted, or minus, not clotted. The ranges of the amounts of anticoagulants that were used were wider than those that are shown.

following it. Sometimes the dog defecated. After recovery from the anesthetic, however, the animals were always normal, active, and hungry.

Table IV shows that carrageenin has anticoagulant activity in human blood, is less potent than heparin, and affects principally the thrombin time.

As the effect of carrageenin upon blood coagulation appeared to be principally antithrombic, this activity was chosen as the basis for quantitative assessment. Figures obtained from these tests are shown in Table V. For each anticoagulant there was a certain range of concentrations in plasma that showed an approximate linear relationship to the thrombin time. On this basis calculations were made from the average figures obtained from the tests on the plasmas of the 3 dogs that had been used to test all of the compounds. The results of the calculations are shown graphically in Fig. 1. The constants that describe the slopes of the lines for heparin and λ-carrageenin, and of the linear portions of those for unfractionated carrageenin and κ-carrageenin, indicate antithrombic activities for the carrageenins, in the order given, of 1/13, 1/24, and 1/34 that of heparin. The curves indicate that, as in the case of the whole-blood clotting time, larger amounts of unfractionated carrageenin or of the κ-fraction do not proportionately affect the thrombin time.

Discussion

The intravenous injection of carrageenin was tolerated by dogs, but there were some adverse effects. Houck, Morris, and Lazaro[15] observed drowsiness in dogs that had been given carrageenin intravenously, and defecation in those that had been given other seaweed polysaccharides by that route.

Carrageenin inhibits peptic digestion. It is uncertain whether the effect is upon enzyme[22] or substrate.[24] Mora and Young[26] observed that polyglucose sulfates precipitated certain basic proteins that act as lytic enzymes at pH below 7, and inhibited this activity. The large organic anions should react with the basic groups of any protein in the system, whether they belong to enzyme or substrate, and thus exert inhibitory activity. For the antienzymic effect the stoichiometric proportions should therefore be important. This is consistent with our observation that λ-carrageenin, with a higher sulfate content, has greater antipeptic activity than κ-carrageenin, and it also fits the observation of Anderson and Watt[28] that among degraded carrageenins antipeptic activity is directly related to the sulfate content.

The principal basis of the anticoagulant activity of carrageenin appears to be, as with heparin, an antithrombic property. The greater activity of the λ-fraction may be related to its higher sulfate content because it has been shown with fractionated heparin that anticoagulant activity increases with the sulfate content.[27]

Bernfeld and co-workers[29] showed that a number of macromolecular polyanions, including carrageenin, will precipitate β-lipoproteins, and that λ-carrageenin forms a more stable complex than does κ-carrageenin. Carrageenin also

Table V. Comparison with heparin of the antithrombic activity in vitro of carrageenin and of its κ- and λ-fractions in human and dog plasma*

Anticoagulant	Subjects	Amount of anticoagulant (μg)								
		0 (saline)	0.1	0.5	1	5	10	20	40	60
		Thrombin time (seconds)								
Heparin	Dogs A, B, C, and D	10	13	23	> 30					
Whole carrageenin	Dogs A, B, C, and D	10	10	11	13	16	20	27	> 30	
κ-Carrageenin	Dogs A, C, and D	10	10	12	13	14	16	19	26	> 30
λ-Carrageenin	Dogs A, C, and D	10	10	11	12	18	27	> 30		
	Human									
Heparin		12	14	> 30						
Whole carrageenin		13	12	13	13	16	21	28	> 30	
κ-Carrageenin		12	13	13	15	16	18	22	24	> 30
λ-Carrageenin		12	13	13	16	27	> 30			

*For dogs the values are averages. The carrageenin was prepared in the laboratory from [illegible]. Measurement of thrombin time: 0.2 ml. citrated plasma [illegible] 0.1 ml. saline plus [illegible] ml. thrombin solution at 37° C.; time from the addition of thrombin to the appearance of fibrin. Anticoagulant was added in the 0.1 ml. of saline in the amounts shown.

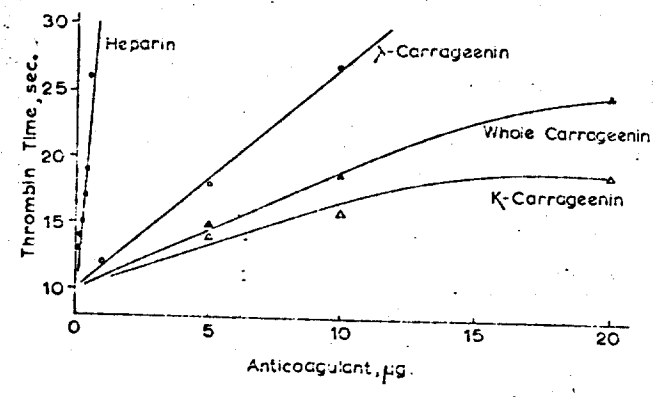

Fig. 1. The antithrombic activity of carrageenin. Data from Table V, with some additional for heparin. The relationships shown are based on average figures for thrombin time in the plasma of dogs A, C, and D, and for 6 levels of heparin (0.1 to 0.6 μg) and 3 levels of each carrageenin. The following equations express a straight line relationship in the case of heparin and λ-carrageenin, and of whole carrageenin and κ-carrageenin up to 10 μg. Heparin, $y = 10 + 24\ x$; λ-carrageenin, $y = 10 + 1.8\ x$; whole carrageenin, $y = 10 + x$; κ-carrageenin, $y = 10 + 0.7\ x$.

stimulates the formation of fibrous tissue,[29] and acts as an antilipemic agent.[19] So far as we are aware, the relative activity of the two fractions in these relationships has not been reported.

To those who are interested in the possible use of carrageenin in the treatment of peptic ulcer, the hazard of placing a material with mild anticoagulant activity in contact with a potentially hemorrhagic lesion must be considered.

We thank Dr. W. Yaphe for the preparation of the κ- and λ-carrageenins in this laboratory, Dr. E. Gordon Young for a critical review of the manuscript of this paper, and Dr. A. C. Neish for helpful suggestions in preparing it.

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NOTES

THE ANTITHROMBIC ACTIVITY OF CARRAGEENIN IN HUMAN BLOOD*

W. W. HAWKINS AND VERNA G. LEONARD

Carrageenin is a sulphated galactan present in the red seaweeds *Chondrus* and *Gigartina*. It has been separated into two components, κ- and λ-carrageenin (1, 2), which differ in some chemical and physical properties (3–6). λ-Carrageenin has a higher sulphate content than κ-carrageenin.

An antithrombic property of the carrageenins has been described (7). On the basis of the amounts required to double the thrombin time in blood plasma, λ-, unfractionated, and κ-carrageenin respectively were about 1/15, 1/25, and 1/62 as active as heparin. Preliminary tests with human blood indicated the same effect, but suggested a quantitative difference (7). This note provides more information on the effect in human blood.

Methods

Carrageenin was prepared from *Chondrus crispus* (Irish moss), and some of it was separated into the κ- and λ-fractions (3). It was compared with U.S.P. heparin sodium (Connaught) with a potency of 100 I.U. per mg.

Solutions, reagents, conditions of the tests, and measurement of the prothrombin and thrombin times were the same as those that have previously been used in this laboratory (7, 8). Measurement of the prothrombin time was modified to include the addition of 0.1 ml of saline. When anticoagulants were added they were incorporated into the 0.1 ml of saline that was put into each system.

Experiment 1 was designed to determine the main site of action of the carrageenins in the clotting process. Prothrombin and thrombin times were measured on three samples of plasma, with the addition to each system of varying amounts of heparin and of the carrageenins. Amounts required to double the normal times were determined.

Experiment 2 was designed to estimate the comparative potencies of the carrageenins as inhibitors of the stage of clotting that they principally affect. The thrombin time was measured on 20 samples of plasma, with the addition of varying amounts of the anticoagulants.

Results

The results of *Experiment 1* are shown in Table I. On the basis of the criterion that was used the carrageenins were 3–4 times as active in prolonging the thrombin time as the prothrombin time. They were less potent

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TABLE I

Amount of anticoagulant required to double the time for appearance of clot* (μg/0.1 ml of plasma)

Anticoagulant	For prothrombin time	For thrombin time
Heparin	3.0	0.15
λ-Carrageenin	10.0	2.5
Whole carrageenin	40.0	7.5
κ-Carrageenin	50.0	15.0

*The figures are averages obtained on three samples.

than heparin, but they resembled it by inhibiting principally the terminal stage of clotting, the transformation of fibrinogen to fibrin. λ-Carrageenin was more active than κ-carrageenin, and the activity of the unfractionated material was intermediate. These results were similar to those that have been obtained with dog blood (7).

The results of experiment 2 are shown in Fig. 1. On the basis of amounts

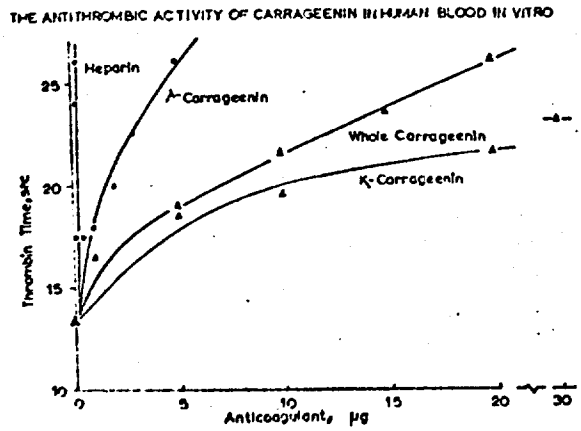

FIG. 1. The antithrombic activity of carrageenins. Abscissa shows μg of anticoagulant added in 0.1 ml of saline to 0.2 ml of citrated plasma and 0.1 ml of thrombin solution (2 units). Each point represents the average figure obtained on 15–20 samples of plasma.

required to double the thrombin time, λ-, unfractionated, and κ-carrageenin were respectively 1/17, 1/67, and <1/167 as active as heparin. The same relationships derived from the figures in Table I were 1/17, 1/50, and 1/100. It is obvious that this method is inadequate for a quantitative assessment of the potency of the more weakly active substances because of the large amounts necessary, and the disproportionality between additional amounts and the increments in antithrombic effect. When the results are compared with those that were obtained with dog blood (7), it is suggested that the carrageenins are less active in human blood.

Comment

Carrageenin has both antithrombic and antipeptic activity (7). It has been shown with degraded carrageenins (9) and with the κ- and λ-fractions (7)

that the antipeptic activity is higher in the materials with higher sulphate content. The greater antithrombic activity of λ-carrageenin is also probably related to its higher sulphate content, because it has been shown with fractionated heparin that anticoagulant activity increases with the sulphate content (10).

There is a point of possible clinical interest in connection with the antithrombic and antipeptic properties of carrageenin. There has been some interest in its use in the treatment of peptic ulcer. Because of its anticoagulant activity the potential danger of this should be realized.

Acknowledgment

Most of the samples of plasma were obtained from the laboratory of Dr. H. C. Read, Consultant Hematologist in the Division of Laboratories, Nova Scotia Department of Health. Dr. Read also critically reviewed the contents of the manuscript of this paper. We offer him thanks for this help.

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CARRAGEENAN AS A DIETARY CONSTITUENT FOR THE RAT: FAECAL EXCRETION, NITROGEN ABSORPTION, AND GROWTH[1]

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Abstract

At levels of 2–20% in the diet of young rats, carrageenan is quantitatively excreted in the faeces.

In diets fed in equal amounts, both cellulose and carrageenan, when substituted for starch in various proportions, affect the growth of young rats. Beginning at levels of 10–15%, however, growth is slower with carrageenan, and there is less absorption of dietary nitrogen.

Carrageenan, like cellulose, has no nutritional value. The depression of nitrogen absorption could explain the slower growth rate that occurs with carrageenan in the diet.

Introduction

Carrageenan is a sulfated galactan that is extractable with water from certain red seaweeds, in particular *Chondrus crispus* and *Gigartina stellata*. It is strongly hydrophilic, and is used in the commercial preparation of some foods. For this reason it is of interest to know how well and in what quantity it is tolerated as a dietary constituent, and whether it has any nutritional value.

Huang and Giese (1) studied the digestibility of a number of algal and other polysaccharides in three marine herbivores, *Strongylocentrotus purpuratus*, *Poggetia producta*, and *Cryptochiton stelleri*. Extracts of stomach and of intestine digested some, but not all, of the substances that were studied and there were differences among animals, organs, and polysaccharides; carrageenan was not digested.

Nilson and Wagner (2) fed to rats and mice diets containing 1–25% carrageenan for up to 2 years, and found no effect on growth or tissues that could definitely be ascribed to carrageenan, although the animals that were fed the largest amount were somewhat lower in body weight. Estimation of the apparent digestibility of the carrageenan during the 10th week indicated a mean value of 50% at all levels of intake but it was admitted that utilization by intestinal bacteria may have complicated the results obtained.

Yaphe (3) has developed a method that can be applied to the quantitative determination of carrageenan, making possible a valid estimation of the faecal excretion of the material fed. An assessment of this important aspect of its nutritional behavior can now be made. Carrageenan has been shown to have antipeptic activity (4–6); consequently, if there is an effect upon growth it might be due to a diminished assimilation of nitrogen. The experiments here described were undertaken to determine the faecal excretion of dietary carrageenan in a monogastric animal, the young rat, and its possible effect upon growth and the absorption of nitrogen. Carrageenan was fed as a constituent of diets containing all essential nutrients and the results were compared to those obtained when cellulose was fed at the same levels.

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Procedure

The carrageenan was a commercial preparation called Seakem, type 6, manufactured by Marine Colloids, Inc., Rockland, Maine. Eleven diets were used, all of which contained 16% casein, 40% sucrose, 4% salt mixture (7), 10% corn oil, and 30% polysaccharide. In one (A) starch was the only polysaccharide; in others cellulose (B–F) and carrageenan (G–K) were incorporated to replace it in varying proportions (to supply 2, 5, 10, 15, and 20% in the diets (see Tables I and II)). The following were added per kg: cod-liver oil concentrate, 2 mg (400 I.U. vitamin A and 100 I.U. vitamin D); α-tocopherol acetate, 30 mg; 2-methylnaphthoquinone, 1 mg; choline chloride, 1 g; inositol, 200 mg; thiamine hydrochloride, 2 mg; riboflavin, 3 mg; pyridoxin hydrochloride, 2 mg; calcium pantothenate, 15 mg; nicotinic acid, 2 mg; *p*-aminobenzoic acid, 1 mg; biotin, 0.3 mg; and folic acid, 0.3 mg.

In all experiments rats were maintained individually in screen-bottomed cages, and allowed water ad libitum. They were weighed at least twice a week. On selected days they were transferred to metabolism cages for 24 hours, and the urine and faeces were collected for analysis, with separation effected by the fitting of an inverted cone screen between the funnel and the mesh floor of the cage. In the metabolism cage, the dish containing food was placed in a tray with an upturned lip in a side compartment that would allow entrance of the animals' head and neck. Very little scattering of food was therefore possible, and any residual food could be readily seen.

Carrageenan in faeces was determined by the resorcinol method of Yaphe (3), and nitrogen in the diets, faeces, and urine by a micro-Kjeldahl procedure.

Experiment 1

Rats were arranged into four groups of three males and seven females each, with average individual body weight of 112 g. They were fed the diets as indicated in Table I. Each rat was fed 10 g of food per day, all of which was consumed. On the 31st day faeces for the 24-hour period were collected from seven rats on each of the regimens I and J, and from four on each of D and E, and the amount of carrageenan in them was determined. The results are shown in Table I.

Experiment 2

Rats were arranged into three groups of five males and five females each, with average individual body weight of 91 g. They were fed the diets as indicated in Table I. Each rat consumed all of the 10 g of food offered per day. On the 23rd day faeces and urine for the 24-hour period were collected from each rat, and the nitrogen content was determined. From the figures thus obtained

TABLE I

Faecal excretion of carrageenan, nitrogen absorption, and growth in the rat (experiments 1 and 2)

Regimen	Polysaccharides in diet, %			Increase in body wt. as % gain over initial		Carrageenan in faeces, per rat per day, % of ingested (range, average, and S.D.) (expt. 1, 31st day)	Nitrogen per rat per day, % of ingested (range, average, and S.D.)	
	Starch	Cellulose	Carrageenan	Expt. 1, 31st day	Expt. 2, 23rd day		Absorbed (expt. 2, 23rd day)	Urinary (expt. 2, 23rd day)
A	30				64		99–100 *100*±0.2	34–72 *52*±11.9
D	20	10		46		0		
E	15	15		45	55	0	91–94 *92*±0.8	44–58 *51*±4.7
I	20		10	48		88–109 *99*±7.3		
J	15		15	46	51	92–114 *101*±9.7	80–87 *84*±2.2	29–51 *48*±2.4

Between	p values for statistically significant differences of nitrogen	
A and E	<0.01	
A and J	<0.01	<0.05
E and J	<0.01	<0.02

TABLE II

Faecal excretion of carrageenan, nitrogen absorption, and growth in the rat (experiment 3)

Regimen	Polysaccharides in diet, %			Increase in body wt. as % gain over initial	Carrageenan in faeces, per rat per day, % of ingested (range, average, and S.D.)	Nitrogen, per rat per day, % of ingested (range, average, and S.D.)	
	Starch	Cellulose	Carrageenan			Absorbed	Urinary
A	30			208		87–97 *93*±2.4	27–84 *55*±11.1
B	28	2		189		89–96 *92*±1.6	40–76 *56*±5.2
C	25	5		176		90–96 *92*±1.6	40–76 *54*±6.8
D	20	10		174		89–99 *92*±1.5	30–77 *52*±7.6
E	15	15		170		90–95 *92*±1.4	42–73 *55*±5.0
F	10	20		137		89–96 *92*±1.6	28–68 *52*±7.4
G	28		2	206	78–114 *96*±9.2	90–98 *95*±1.4	39–77 *49*±6.9
H	25		5	183	78–111 *93*±7.7	86–95 *91*±2.2	37–70 *53*±6.7
I	20		10	152	78–115 *91*±8.6	87–93 *90*±1.9	33–72 *52*±6.8
J	15		15	138	79–101 *92*±8.2	82–97 *89*±2.2	25–67 *44*±7.0
K	10		20	101	79–107 *89*±7.4	82–95 *88*±2.3	21–64 *43*±9.6

The following tables are in the N.R.C. depository: Table III, p values for statistically significant differences of nitrogen values in Table II; Table IV, the faecal excretion of carrageenan, nitrogen absorption, and growth rate in the rat after 27, 98, and 148 days (summarized in Table II); and Table V, p values for statistically significant differences of nitrogen values in Table IV.

the amount of ingested nitrogen that was absorbed was calculated. The results are shown in Table I.

Experiment 3

Rats were arranged into 11 groups of five males and five females each, with average individual body weight of 90 g. They were fed the diets as indicated in Table II. Each rat was offered, and consumed, 8 g of food for the first 6 days, 10 g for the next 6 days, 12 g for the next 100 days, and 15 g per day thereafter. On the 27th, 98th, and 148th day faeces for the 24-hour period were collected from each rat on the diets containing carrageenan, and the amount of carrageenan in them was determined. On the next day, faeces and urine for the 24-hour period were collected from each rat in the experiment to determine their nitrogen content and subsequently calculate the amount of the ingested nitrogen absorbed. The results are summarized in Table II. Complete data in three other tables (III, IV, and V) are available.*

Results and Discussion

The results of the experiments recorded in Tables I and II show that when carrageenan is fed to rats at dietary levels of 2–20% it is 90–100% excreted in the faeces. It is therefore not digested, and consequently cannot have any direct nutritional value.

The figures show that cellulose in the diet at levels of 2–20%, as a substitute for starch, decreases the rate of growth when the diets are fed in equal amounts. The figures also indicate that it may slightly decrease the absorption and the urinary excretion of nitrogen.

When carrageenan instead of cellulose is fed in the same amounts under the same conditions, there is a greater adverse effect upon growth and the absorption of ingested nitrogen, and a definitely smaller urinary excretion of nitrogen. This becomes more evident as the proportion of carrageenan in the diet is increased. The decrease in absorption of nitrogen could explain the greater retarding effect of carrageenan than of cellulose upon growth. When starch was the sole polysaccharide of the diet, an average of 38% of the ingested nitrogen was retained. When cellulose and carrageenan were substituted, the average figures for the retention of ingested nitrogen were 39 and 42% respectively. The latter, presumably, was not of sufficient magnitude to compensate for the decreased absorption at the prevailing caloric level.

Throughout these experiments, there was no indication of an adverse effect of carrageenan at the levels fed upon the health of the rats, with the possible exception of the effect of levels of 10–20% upon the rate of growth. The effect upon the absorption of nitrogen was not significant below such levels. It is improbable, therefore, that levels below 10% in a nutritionally adequate diet under any circumstances, even if fed continuously, would show an adverse

*Tables III, IV, and V have been placed in The Depository of Unpublished Data, National Science Library, National Research Council, Ottawa, Canada, and photocopies may be obtained upon request to the Depository.

effect, particularly if food were offered ad libitum. It is even less likely that the small amounts in certain human foods would have any deleterious effect.

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Work done by the "Laboratoires de Recherches Continental Pharma, Brussels and the "Laboratoire d'Anatomie Pathologique" of the Universite Libre de Bruxelles (Prof. P. Dustin)

USING CARAGEEN TO TREAT EXPERIMENTAL ENTERITIS

by G. Lambelin, G. Mees, M. Foster and R. Parmentier

Carrageen "Carragenine" is principally known for its therapeutic effect on gastroduodenal ulcers (3). Its mode of action is still interpreted variously and although it is known that it has antipeptic properties in vitro and in vivo (1, 6, 9, 13, 17) , recent papers indicate (4 and 6) that it is capable of decreasing the acid secretion and forming a protective complex with the mucus. Furthermore it appears that its physical properties can play an important role. One of us has indeed noted (12) that for identical sulphate contents the protective power of the carrageen (Carragenine Continental Pharma, quality A8, abbreviation adopted: carragenine A8) with regard to experimental ulcers increases in parallel manner to the viscosity of its solutions.

In view of these various elements, it was interesting to determine whether the product was also capable of protecting the intestinal mucosa beyond the duodenum. With this in mind we treated an experimental ulcerative enteritis of the rat with carrageen and we had compared the activity of carrageen with that of bismuth subnitrate. At the same time by an appropriate method we attempted to determine to what point of the digestive tract the carrageen could be found in combination with the mucus.

Materiel and methods

Three batches of male albino rats of the Wistar source with an average weight of 150 grams were used.

I. Revelation of the carrageen in the intestine

The tests involved 52 animals. The rats, deprived of food for 24 hours, were given the carrageen by esophagus sound. Corresponding to each rat treated in this manner there was a control rat receiving an equal volume of physiological water through the same way (esophagus).

The animals were sacrificed at different intervals and fragments of the stomach and intestine were removed which were immersed in methyl alcohol for 24 hours. Once the fixing was complete the fragments were colored with phenic toluidine blue after passing through water and acetic acid at 1%.

The metachromatic properties of the carrageen which remain after acidification appear with a red-violet coloring localized at the surface of the mucosa and clearly different from the blue coloring observed in the control animals.

2. Protective effect of the carrageen on the intestine

A sdrious enteritis is produced by the toxic doses of 1-(p-chlorobenzoyl)-2-methyl-5-methoxyindole-3-acetic acid (= compound 1) administered in its marketed form.

The test was conducted on a batch of 60 rats divided into three groups: the first group of 20 rats is the control group. These animals are given a daily dose of compound 1 of 10 mg/kg by esophagus probe in a physiological solution; the second group of 20 rats is treated in the same way by the compound I, but also receives two daily doses of 200 mg/kg of carrageen A8 in solution at 2%. The third group, besides the treatment with compound I, receives two daily doses of 200 mg/kg of light bismuth subnitrate.

The experiment is carried out for five days. The surviving animals on the fifth day are sacrificed.

The autopsy of all the rats is done by giving particular attention to the digestive tract where the appearance of the intestine at the level of its various segments is carefully noted. Systematic samplings of the duodenum, jejunum, ileum, cecum and colon were taken for histo-pathological examination. After Bouin fixation the cuts were colored with the usual hematoxylin-cosine.

3. Determination of the lethal doses

The DL_{50} were determined for compound 1 administered separately and in conjunction with carrageen or the bismuth subnitrate using the method of Litchfield and Wilcoxon (10). We used 90 rats distributed into three groups:
group I: administration of a single dose of compound I to five batches of six rats. The doses selected varied from 5 to 80 mg/kg; group II: treatment at the same doses by compound I along with two administrations of carrageen A8 (200 mg/kg); group III: same treatment with compound I along with two administrations of bismuth subnitrate (200 mg/kg).

The period of observation of the subjects was set at 10 days.

RESULTS

1. Presence of carrageen in the intestine

As we could have expected, the carrageen crosses the digestive tube without degradation .

By the method of coloration used we can follow its path all along the digestive tract according to a very strict schedule from the stomach to the large intestine. This progression was summarized in table I.

2. Experimental enteritis

a. Macroscopic study

Considerable intestinal lesions are present in all of the control animals (plate I, fig. 1 and 2). They are already observed in the animals which succumbed the earliest after two days of administration of compound I.

Although the stomach appears free of any change, the small intestine presents an intense congestive appearance, particularly in its distal part.

Necrotic regions characterized by a yellowish coloring are evident in 16 cases out of 20. The large intestine is also affected, but only at the cecum. These lesions are always accompanied, even in the animals who died the earliest, by inflammatory reactions at the peritoneum which is thickened and covered with purulent blurs lining all of the abdominal viscera in the most serious cases.

Upon opening the intestine the mucosa is of a wine color and the content

TABLE I

DETERMINATION BY METACHROMASIA OF CARRAGEEN A8 AT VARIOUS LEVELS OF THE DIGESTIVE TRACT OF THE RAT

a Dose de Carragénine A8 (mg/kg)	b Intervalle entre administration et prélèvement	c Localisation de la coloration métachromatique				
		d Estomac	e Jéjunum	f Iléon	g Caecum	h Colon
100	1 heure i	+	0	0	0	0
100	2 heures	+	+	+	0	0
100	4 heures	+	±	±	±	±
200	1 heure	+	0	0	0	0
200	2 heures	+	+	±	0	0
200	4 heures	+	+	+	±	±
400	1 heure	+	+	0	0	0
400	2 heures	+	+	+	0	0
400	3 heures	±	0	+	0	0
400	4 heures	+	+	+	+	+
400	6 heures	0	0	0	0	0
400 × 2*	1 heure*	+	+	+	+	±
400 × 2*	2 heures*	+	+	—	+	+

a= dose of carrageen A8 /mg/kg)

b= interval between administration and sampling

c= location of the metachromatic coloration

d= stomach

e= jejunum

f= ileum

g= cecum

h= colon

i = heure = hour heures= hours

*The two treatments are done with 4 hours of interval. The number in the 2nd column therefore gives the time elapsed between the second treatment and the sampling.
Note: For each dose and for each time contemplated, the tests are done on two rats compared with two control rats.

PLATE I

Fig. 1 and 2. Effect of compound I. The ulcerative enteritis involves primarily the terminal part of the small intestine and the cecum which are covered with purulent blurs. Note the intense congestive phenomena. Upon opening (Fig. 2) , the mucosa has a hemorrhagic appearance and presents numular ulcerations.

Fig. 3 and 4. Inhibition of the effect of Compound I by carrageen A8. Definite disappearance of the congestive situation. Presence of purulent exudates on the peritoneum at the level of the terminal ileum. Upon opening (Fig. 4), the mucosa is of considerably normal coloring, but we note the presence of less extensive ulcerations.

Fig. 5 and 6. Inhibition of the effect of Compound I by the bismuth subnitrate . The black coloration observed is due to the presence of the product in the mouth of the intestine and it disappears after washing , as shown by the open segment (Fig. 6). Presence of purulent blurs on the peritoneum at the cecum. The mucosa, which is congestive in places, presents ulcerations comparable to those observed after carrageen treatment.

is sometimes definitely hemorrhagic as in infarction. After washing we can observe ulcers of variable size but becoming more and more numerous at the level of the terminal part of the jejunum and all the ileum.

Examination by transparency confirms that in many places these ulcers can be perforated in the peritoneal cavity where they are most often clogged by epiplaic flanges or couplings of the serous membrane.

When the animals treated with compound I receive at the same time carrageen (Plate I, fig. 3 and 4) , the intensity of these lesions is clearly smaller.The congestion is less marked or sometimes absent. The number of cases of peritonitis diminishes. Although it is constant in the animals of the first group,it is observed in 14 out of 20 subjects of the second group.

Upon opening of the intestine the appearance of the mucosa is also different. The distinctly inflammatory appearance often disappears and the mucosa often retains its normal coloration. The protective power of the carrageen, however, is not total because ulcers are still visible but in small number.

The result is comparable in the group protected under the same conditions by the bismuth subnitrate (Plate I, fig. 5 and 6).

The inflammatory lesions as a whole are less clearly attenuated and the ulcerative condition remains in a similar proportion of cases. The peritonitis is evident in 13 cases out of 20.

b. Microscopic study

The intestine is studied at all levels by transverse (cross-sections) cuts from the stomach to and including the cecum. About 20 cuts (sections) are done per animal and this permits a systematic determination of all of the segments. With a few rare exceptions, all of the examinations show lesions at one or another level. But it is difficult to estimate their seriousness because of their dispersion and their focal nature.

As a whole, the results agree with those of the macroscopic examination. In order to make the examination as objective as possible the sections were read while not informing the observer of the treatment applied. In no case was there gastric lesion. The changes in the mucosa did not start to become evident until from the duodenum in the most serious cases.

In the control animals treated solely by the compound I the lesions of the small intestine present a frequency and an intensity greater than those observed in the animals of the two other groups. They are characterized by the habitual changes in the inflammation: congestion, leucocyte infiltration, necrosis and ulceration. The last two characteristics are particularly marked.

The sequence of these lesions can be followed all along the digestive tract (Plate II). The initial phenomenon is most often limited to the extremities of the villosities where the glandular structures disappear gradually whereas the infiltrate becomes more and more abundant. Finally a veritable amputation occurs with elimination of the villosities at the mouth of the intestine.

The necrosis which gradually extends itself ends up by involving large regions of the mucosa which sometimes ulcerates over large expanses. Most often this ulcerative characteristic is focal and a distinct border is created from one part to the other of the necrosed region between the changed mucosa and the healthy mucosa.

In the most intense manifestation all of the wall can be involved and necroses as a whole, creating a perforation neatly. At this stage one frequently finds fibrinoid at the mouth of the capillaries.

The necroses can occur at any part of the intestine but are found rather frequently in the direction of the mesenteric insertion. It should be noted that the Peyer plates do not show any elective attack and that they are only secondarily undertaken after the overlying mucosa has been initially abrased.

Plate II.

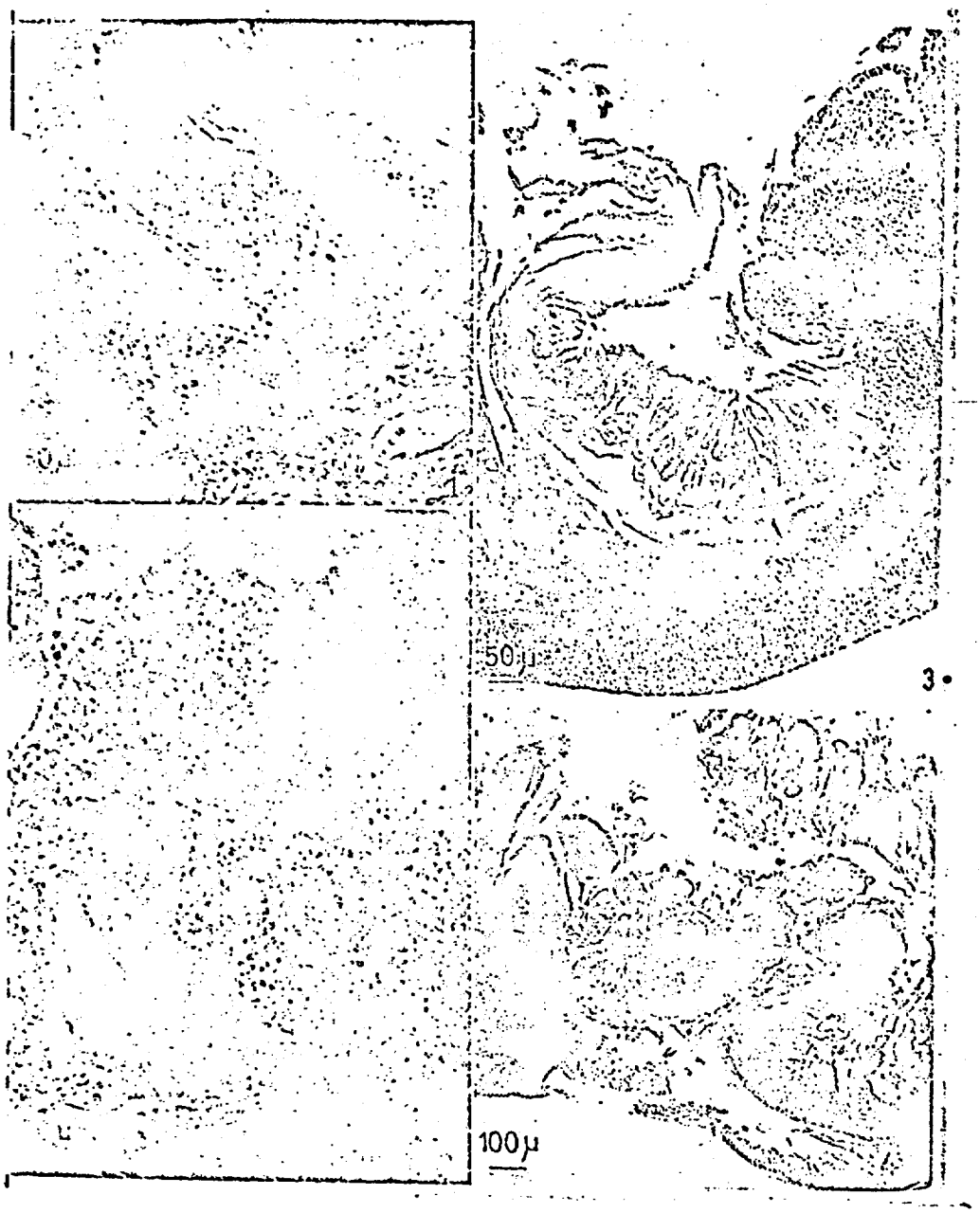

Fig. I. Lesion of initial necrosis marked by the obliteration of the villosities and th disappearance of the glandular structures. Only remaining are the bottom of the crypts of Lieberkuhn and the connactive tissue is widely infiltrated with leucocytes (aqueous Bouin, hematoxyline-cosine).

Fig. 2. Lesion already more advanced. To the right the surface regions of the mucosa assume a ghost-like appearance. To the left the mucosa of which there only remains a portion of gland still presents a granulomatose appearance (aqueous Bouin, hematoxyline-cosine).

Fig. 3. The lesion has become essentially necrotic. The initial surface attack is of a focal nature. The sub-mucosa and the internal muscular layer are already clearly attacked. Notice the precise limit of the lesion (aqueous Bouin, hematoxyline-cosine).

Fig. 4. Terminal phase of the alceration announcing the perforation: necrosis neatly involving the entire extent of the wall. Amputated villosities are liberated in the mouth of the intestine (aqueous Bouin, hematoxyline-cosine).

In the 20 animals examined the peritonitis is constant and sometimes very marked, most often around the segments which are the most distal from the small intestine.

Both in the group receiving carrageen treatment and in the group receiving bismuth treatment the congestive phenomena are less manifest although the inflammatory exudates remain just as abundant at the ulcers. As a whole, furthermore, these ulcers are less numerous and the necrosis less extensive. On three occasions and in each of the two groups the microscopic examinations reveal no lesion.

3. Determination of the lethal doses

a. Compound I alone: LD50 = 10.4 mg/kg.
Confidence limits 95% : 5.2-20.8 mg/kg.

b. Compound I + carrageen A8: LD_{50} of compound I:15 mg/kg.
Confidence limits: 95%: 9.7-29.7 mg/kg.

c. Compound I + bismuth subnitrate:
LD_{50} of compound I:14 mg/kg.
Limits of confidence: 95%: 7-28 mg/kg.

There is no significant difference between the three results.

DISCUSSION

Determination of the carrageen in the intestine

The metachromatic properties of the carrageen were used by Marcus and Watt (16) to determine the effect of the product in the digestive tract.

The appearance of the red coloring after the action of the phenic toluidine blue on a mucosa previously washed and acetified indicates a very great adherence of the polysaccharide to the mucosa attributed to a connection to the mucus. We will also note that in the stomach this metachromasia does not occur at the rumen but at the glandular portion which is rich in mucus. Although the quantitative detection and consequently the determination of the therapeutic dose are not possible by this method, it does permit reaching the conclusion that the carrageen A8 forms an active complex both with the intestinal mucus and with the gastric mucus. This complex can be revealed up to the level of the colon as long as the doses are sufficient.

Production of the experimental enteritis

The advantage of the toxic enteritis is that it can be reproduced at will at intensities which can be controlled and compared. After having unsuccessfully tried drastic purgatives such as croton oil (administered orally or rectally) we used the anti-inflammation agents or anti-rhumatismal agents. It is known that these products, as long as they are given at sufficient doses, are capable of causing an inflammation of the digestive tube, particularly at the level of the stomach. This form of toxicity is well known for the corticosteroids (9 and 15), phenylbutazone (6), acetylsalicylic acid (14) and cincophen (7 and 11). Vecchio describes for the indomethacine of the gastric ulcers and of the peritonitis (19). Using the marketed form of this last product we observe that the intestinal lesions are great, whereas the gastric lesions become negligible.

The enteritis achieved under the conditions described reaches the terminal part of the small intesting and the cecum. It is present in all of the control rats and is characterized by focal ulcerations and deep parietal necroses,

accompanied by peritonitis. The mortality is great.

The etiology of the digestive lesions caused by the anti-inflammatory agents is poorly known. It has been thought that there could be a correlation between their capability of inhibiting the synthesis of the muco-polysaccharides in vitro (5, 20, 21, 22) and the capability of causing changes at the digestive tract. This correlation appears to be small and Anderson (2) believes that one should rather seek out the cause of the lesions in the inhibiting action of the anti-inflammatory agents on the succinic oxydase system or on a certain point of the succinate cytochrome chain.

Action of carrageen A8 with respect to the experimental enteritis

Treatment with carrageen A8 decreases the incidence and magnitude of the cases of ulcerative enteritis and at the same time the cases of peritonitis. Its effectiveness is comparable to that of the bismuth sub-nitrate. The toxicity of compound I is not changed by the treatment. We studied this question in particular. It was necessary to find out whether the action of carrageen A8 could be explained by an adsorption of the compound I and consequently an inhibition in its inflammatory action on the intestine. It is quite evident that if this is the case then the toxicity of compound I must be smaller. The results which we obtained in the toxicity tests showed us that this hypothesis should be rejected. Indeed, treatment with carrageen A8 does not reduce the mortality.

The product thus seems to act by choice in protecting the intestinal mucosa. Its mode of action is obviously not defined, but we can assume the formation of a mucus-polysaccharide complex comparable to that which is called upon to explain the effectiveness of the product in the treatment of gastric ulcer.

Everything leads one to believe that a protective effect of the carrageen A8 can also occur beyond the small intesting and be beneficient from there on in some pathological conditions such as ulcero-hemorrhagic colitis. Up to the present time it has not been possible for us to reproduce comparable conditions in the animal.

The Lancet 2: 414-15, Aug. 22, 1970

CARRAGEENAN: EFFECTS IN ANIMALS

SIR,—In response to the suggestion of Marcus and Watt[1] that carrageenan may cause intestinal lesions in certain animals, we have conducted a multicentre study of its effects in guineapigs, rats, and mice. We should mention that earlier work on animals produced no evidence of such an effect: no gastrointestinal lesions were noted by Nilson and Wagner,[4] who fed carrageenans (in proportions of 1 to 25%) throughout the lifespan of rats, nor by Hawkins and Yaphe[5] or workers at Paris.

Marcus and Watt offered guineapigs carrageenan solution ad libitum in lieu of ordinary drinking water, and found that they consumed not more than 1·5 kg. per day of the native preparation and not more than 2 g. per day of the degraded preparation. In contrast, our guineapigs consumed between 4 and 5 g. of the degraded product daily. At these levels we did find mucosal erosions, but no ulceration, in the cæca. In rare instances these erosions extended into the colon. The lesions were quite different from those of ulcerative colitis in man. In addition, we found that the drinking solutions were heavily contaminated by micro-organisms.

When rats were allowed to drink solutions of carrageenan ad libitum, we found, unlike Marcus and Watt, that they consumed up to 16·5 g. per kg. per day. Nevertheless, even at this dosage we noted only moderate congestion of the cæcal mucosa, and there was no evidence of erosions.

Further detailed investigations were done in our three laboratories, in which native and degraded carrageenan were given by gavage so that dosage could be more strictly controlled. Administration of both native and degraded carrageenan to 200 rats for 28 days, at doses ranging 70 mg. to 4 g. per kg. per day, produced no histopathological or other changes attributable to this treatment. One of us (S. B.) extended the treatment of his rats to 6 months at a dosage of 4 g. per kg. per day; during this period the rat grew normally, and no evidence of toxicity was noted when they were killed.

M. M. has conducted a 6-month study on mice using the same range of doses, and there has been no evidence of any pathological changes in the large intestine or elsewhere attributable to carrageenan.

The results of the guineapig studies suggest that they are peculiar to this species, and their significance in man is not clear.

Our findings were reported to a meeting on carrageenan organised by the British Industrial Biological Research Association (BIBRA) at the Royal Society of Medicine on Jan. 27, 1970, and have also been reported to the Food Additives and Contaminants Committee.

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We should like to thank Dr. G. B. Shirlaw, Laboratoires Glaxo, Paris, for supplying the native and degraded carrageenan used in the multicentre trial.

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Letters to the Editor

SEAWEEDS AND ULCERATIVE COLITIS IN LABORATORY ANIMALS

SIR,—In recent years considerable interest has been shown in sulphated polysaccharides and their anti-peptic and anti-acid gastric secretory properties—e.g., chondroitin sulphate, heparin, sodium polyanhydromannuronic acid sulphate, and carrageenan.[1-5] These substances are known to protect against various forms of experimental peptic ulceration.[1 6-9] On the continent of Europe, degraded carrageenan derived from red seaweed is used in the treatment of gastro-duodenal ulceration.[10] In the U.K. several clinical trials of degraded carrageenan have been made, and the results of one such trial have been published.[11] Non-degraded or native carrageenan, however, is present in aqueous extracts of the dried red seaweed, *Chondrus crispus*, which is readily obtainable at health-food stores and recommended for various disorders. As yet there have been no reports of any adverse effects—clinical or experimental—resulting from the oral administration of these substances.[12]

We have studied the effects of adding degraded carrageenan (obtained from the red seaweeds, *Chondrus crispus* and *Eucheuma spinosum*, and degraded by mild acid hydrolysis) to the drinking-water of animals on an otherwise normal diet. We observed loss of weight within two to three weeks. Our first observations were made in guineapigs,[13] but subsequently loss of weight was also noted in rabbits, some of which had blood and mucus in the stools. In rats and mice no significant weight-loss was observed. In contrast to our findings with degraded carrageenan, animals fed the non-degraded preparations appeared to be healthy and gained weight satisfactorily.[14]

Investigation, however, revealed that in all four species there were changes in the mucosa of the cæcum, colon, or rectum resembling those found in ulcerative colitis in man. The early changes included pin-head-sized hæmorrhagic and ulcerative lesions, along with cellular infiltrates and crypt abscesses (figs. 1–3). In later stages there was loss of mucosal folds, and in some animals there was scarring of the bowel which led to formation of constriction rings (fig. 4). In mice, the changes were minimal in comparison with the other species, and comprised mainly focal cellular infiltrations of the colonic mucosa.

These changes have been observed in animals fed carrageenan daily from 30 days to 1 year. In the rabbit,

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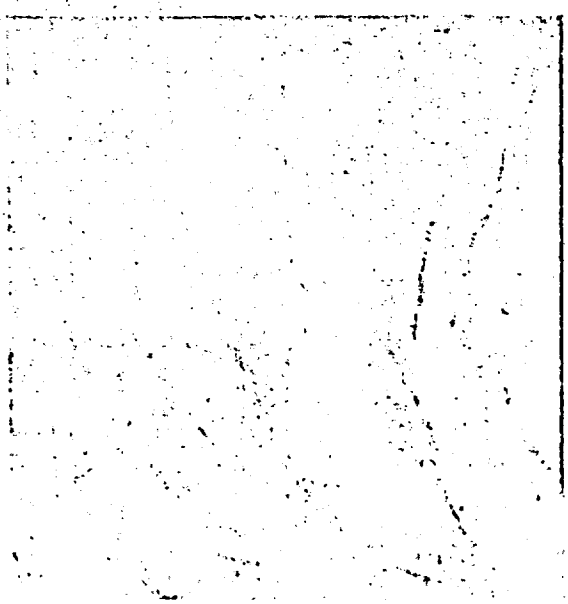

Fig. 1—Hæmorrhagic and ulcerative lesions in cæcum of rabbit fed with carrageenan (×2·5).

Fig. 2—Ulceration of colon of guineapig fed with carrageenan.

The ulcer has penetrated the muscularis mucosæ: the ulcer base shows inflammatory-cell infiltration. Hæmatoxylin and eosin (×56).

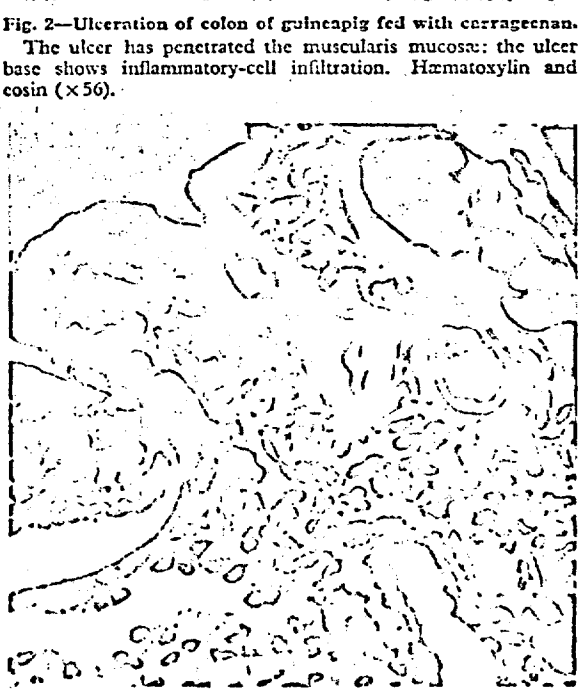

Fig. 3—Ulceration of colon of guineapig fed with carrageenan.

Section from ulcer margin showing a crypt abscess and infiltration of the mucosa by macrophages, lymphocytes and polymorphonuclear leucocytes (×350).

Fig. 4—Ulceration of colon in rat fed with carrageenan.
Note the constriction of the cæcum due to fibrous scar formation at the site of an ulcer.

lesions occurred when degraded carrageenan was added to the drinking water in a concentration as low as 0·1%. The highest concentrations used in our studies were 5% degraded carrageenan and 1% undegraded carrageenan.

These observations may be important in view of the increasing use of carrageenan and other seaweed derivatives in food or food substitutes, and the increasing incidence of ulcerative colitis in man.[15 16]

Supplies of undegraded carrageenan were purchased from J. W. Cumming & Son, Ltd., Salford, and from local health-food stores. Degraded carrageenan, retaining about 29% sulphate, was kindly supplied by Laboratoires Glaxo, Paris.

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Hydrogen ion concentration
Pepsin
Polysaccharides
Proteins
Sulfates

A Study of the Mechanisms of Inhibition of Peptic Proteolysis by a Sulphated Polysaccharide

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The hydrolysis of protein substrates by pepsin is inhibited by sulphated polysaccharides [6, 12, 13, 16, 17, 18]. This inhibition probably explains the protective effect of sulphated polysaccharides in experimental ulcer in animals [3]; it is the basis of the treatment of peptic ulcer in man with various polysaccharides [6, 7, 8]. Furthermore, the existence in the mucosa and gastric juice of polysaccharides and sulphated glycoproteins has recently been demonstrated [10, 11, 14, 15]; it is possible that these sulphated macromolecules contribute, by inhibition of peptic proteolysis, to the resistance of the gastric mucosa to digestion by the gastric juice.

It is thus important to understand the mechanism by which these sulphated polysaccharides inhibit peptic proteolysis. This inhibition has been explained by two different mechanisms: (1) an inhibition of the enzyme itself by denaturation or competition [1, 12], (2) an inhibition by formation of a complex between the polysaccharide and the substrate, which thus becomes resistant to peptic proteolysis [2, 16, 17].

The demonstration by paper electrophoresis or agar diffusion of a complex between polysaccharide and protein substrate (without apparent reaction between polysaccharide and pepsin) enabled us in previous publications [5, 13] to prove indirectly the second theory. We have sought to confirm the latter in a more direct way by studying changes in the rate of the pepsin substrate reaction in the presence of variable concentrations of sulphated polysaccharides.

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Technique

(1) Reagents

(1) The *pepsin* used is commercial Worthington twice crystallised pepsin, 2.5‰ in hydrochloric acid at pH 2.00 on the same day as the experiment.

(2) The *haemoglobin* is a denatured haemoglobin standardised as enzyme substrate (N.B.C.). The substrate is brought to the desired concentration on the day of the experiment by dilution and adjustement to pH 2 of a 5% mother solution in distilled water, prepared according *Chariot* [9].

(3) The *sulphated polysaccharide* used is a degraded product of Carrageenan (C-16 Glaxo-Evans). It is prepared in the desired concentration and its pH is brought down to 2 with normal hydrochloric acid.

(4) The *titrating solution* is 0.05 N hydrochloric acid.

(2) Apparatus

It consists of an Automatic Titration Apparatus Radiometer (Titrator TTl c, Titrigraph SBR 2 c, Autoburette ABU 1 b) and a water Thermostat. The Autoburette is equipped with a microsyringe of 0.25 ml capacity. The titration is carried out in a 5 ml beaker at constant temperature. The pH is recorded with two micro-electrodes (G 2222 C and K 4112 Radiometer).

(3) Principle of the Reaction

An increase in pH value is the result of the consumption of Hydrogen ions associated with the breakdown of peptide links in acid medium. The pH stat method consists in compensating the pH variation by addition of a measured quantity of the titrating solution. The output of titrating Hydrogen ions measures the rate of the enzymatic reaction.

(4) Method

(1) The following conditions were adopted for the reaction:

Volume for the reaction: 2.5 ml.

Reaction medium is composed by: –2 ml of the acid solution of Haemoglobin; –0.5 ml of pure Hydrochloric acid solution at pH 2.00 or 0.5 ml of C-16 polysaccharide solution at variable concentration in Hydrochloric acid at pH 2.00.

Temperature: 37° C.

pH stat: 2.0.

Maximum rate of flow in the autoburette: 0.075 ml/minute.

Proportional band of response in the pH stat system: 0.1 units pH.

(2) The reaction volume was placed in the thermostated beaker at constant temperature for 10 minutes to obtain a thermal equilibrium. The pH stat device was then started up in order to adjust the pH to exactly 2.00. The adjustment of pH should require only a few microlitres of the titrating acid solution in order not to modify seriously the volume of the reaction. When the adjustment is obtained, Pepsin is added in the medium in a very small volume (20 microlitres, i.e. 50 micrograms of pepsin) in order not to modify either the pH or the temperature.

(3) A few seconds are usually sufficient for the output of the burette to become stabilised at a constant value. This is maintained for a variable time (usually a few minutes), which depends on the quantity of free substrate present in the reaction medium. Graphic registration enables one to calculate easily this constant output, which is expressed in microequivalents of Hydrogen ions delivered per minute.

(4) Between two experiments it is essential to rinse the electrodes and the receptacle with 0.1 N. Sodium Hydroxyde in order to denaturate irreversibly the pepsin which might remain absorbed on the surface of the glass. This is followed by several rinses with distilled water.

Results

(1) Variations in the Output of the Titrating Solution in Relation to the Concentration of the Substrate

A series of 41 experiments has been carried out under identical conditions with the same concentration of pepsin (20 micrograms/ml in the reaction medium) but with varying concentrations of substrate (between 2.0 and 20.0 mg/ml in the reaction medium). It enables one to draw up the relationship between the output of the titrating solution and the concentration of the substrate. These ratios are summarized in Table I.

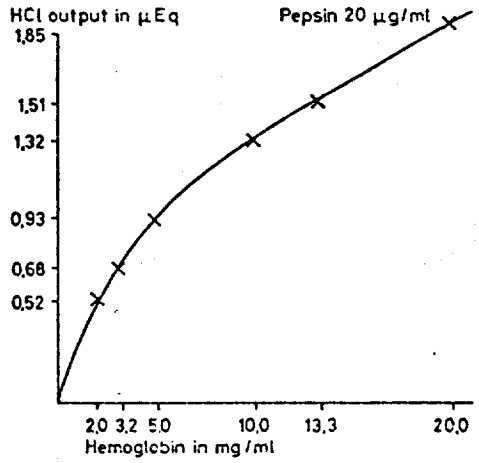

Fig. 1. Variations of the output of titrant HCl in function of Hemoglobin concentration. The graph allows to compute the concentration in "effective" Hemoglobin out of the output of titrant HCl in presence of an inhibitor.

TABLE I

Variations of the velocity of enzymatic reaction (measured by the mean output of titrant HCl) in function of hemoglobin concentration in the medium of reaction (Pepsin concentration is 20 μg/ml).

Substrate Hemoglobin (mg/ml)	No. experiments	Mean output of HCL (μEq/ml)	Standard error
20.0	4	1.89	0.05
13.3	12	1.51	0.05
10.0	5	1.32	0.07
5.0	4	0.93	0.03
3.2	10	0.68	0.05
2.0	6	0.52	0.03

(2) Changes in the Output of the Titrating Solution with Addition of C-16 Sulphated Polysaccharide

These changes have been studied for three different concentrations of substrate: 20, 10 and 5 mg/ml in the reaction medium. The results obtained with varying concentrations of inhibitor are summarised in Tables II, III and IV. The results show that inhibition produced by a given concentration of inhibitor is inversely proportional to the concentration of the substrate.

TABLE II

Variations of the velocity of enzymatic reaction (measured by the mean output of titrant HCl) in function of the concentration of inhibitor polysaccharide in the medium of reaction (Pepsin concentration is 20 μg/ml Hemoglobin concentration is 20 mg/ml).

Inhibitor (mg/ml)	Substrate Hemoglobin (mg/ml)	No. experiments	Mean output of HCL (μEq/ml)	Standard error	% inhibition
0	20	4	1.89	0.05	0
0.4		4	1.84	0.07	2.7
0.8		4	1.82	0.04	3.7
1.6		4	1.67	0.15	11.6
2.0		4	1.61	0.09	14.8
2.8		4	1.51	0.08	20.1
4.0		4	1.30	0.08	31.2

TABLE III

Variations of the velocity of enzymatic reaction (measured by the mean output of titrant HCl) in function of the concentration of inhibitor polysaccharide in the medium of reaction (Pepsin concentration is 20 μg/ml. Hemoglobin concentration is 10 mg/ml).

Inhibitor (mg/ml)	Substrate Hemoglobin (mg/ml)	No. experiments	Mean output of HCL (μEq/ml)	Standard error	% inhibition
0	10	5	1.32	0.07	0
0.4		4	1.26	0.06	4.5
0.8		4	1.19	0.06	9.8
1.2		4	1.13	0.04	14.4
1.6		4	1.06	0.06	19.7
2.0		4	0.95	0.06	28.0
2.8		4	0.80	0.07	39.4
4.0		4	0.48	0.08	63.6

TABLE IV

Variations of the velocity of enzymatic reaction (measured by the mean output of titrant HCl) in function of the concentration of inhibitor polysaccharide in the medium of reaction (Pepsin concentration is 20 μg/ml. Hemoglobin concentration is 5 mg/ml).

Inhibitor (mg/ml)	Substrate Hemoblobin (mg/ml)	No. experiments	Mean output of HCL (μEq/ml)	Standard error	% inhibition
0	5	4	0.93	0.03	0
0.4		4	0.80	0.04	14.0
0.8		4	0.67	0.02	28.0
1.2		4	0.57	0.06	38.7
1.6		4	0.43	0.10	53.8
2.0		4	0.28	0.11	69.9

Discussion

(1) The Technique of Measurement of Peptic Proteolysis

Under the conditions which we have used the technique of the pH stat enables one to measure with satisfactory accuracy the velocity of the enzymatic reaction and its variations due to the presence of an inhibitor.

It is, however, not possible to admit without verification that the velocity of the enzyme reaction is proportional to the output of the titrating solution. In a certain range of pH (i.e. the "Proportional Band" fixed under our experimental conditions at 0.1 pH units) the output of the titrating solution is related to the difference between the fixed pH and the true pH. The output of the burette is thus not proportional to the rate of the enzyme reaction unless the latter is constant (reaction of order zero) for a sufficiently long time. This condition is not fulfilled in the case of low concentrations of substrate or high concentrations of inhibitor. This probably explains why the graph of 1/v in relation to 1/s or of v in relation to v/s does not verify *Michaeli's* and *Menten's* equation in our experiments.

The technique used can thus not be recommended unreservedly as a technique of measurement of the rate of peptic proteolysis. It enables one, on the other hand, to obtain satisfactorily reproducible results when measuring the changes connected with quantitative or qualitative changes in the reaction medium.

(2) Inhibition of Peptic Proteolysis by the C-16 Sulphated Polysaccharide

The inhibition produced by a given quantity of inhibitor is expressed as a percentage of diminution of the titrating solution output. This inhibition is greater when the concentration of the substrate is smaller. This result is consistent with the theory of "protection" of a quantity of substrate proportional to the quantity of inhibitor present in the reaction medium.

This theory seems to be confirmed by calculation of the quantity of substrate apparently "protected" by a unit weight of inhibitor. To do this we have calculated graphically for each experiment carried out in the presence of an inhibitor the concentration of substrate which corresponded in the absence of an inhibitor to the output of titration solution observed in the inhibited reaction. The difference between the "true" concentration and the "apparent" concentration of the substrate enables one to calculate the "protected" concentration and to express it in relation to the concentration of the inhibiting polysaccharide.

The results summarized in Table V show that the quantity of substrate "protected" by 1 mg of C-16 polysaccharide is practically independent of the concentration of substrate and of the inhibitor in

TABLE V

Calculation of the Amount of Hemoglobin that is Protected by 1 Milligram of Polysaccharide C-16

The amount of the "protected" Hemoglobin in every experiment has been computed in establishing the difference between the hemoglobin really present in the reaction, and the "effective" Hemoglobin. The "effective" Hemoglobin is the amount of Hemoglobin which, in the absence of an inhibitor, would make out the same enzyme reaction velocity as an inhibited reaction. The amount of Hemoglobin protected by 1 milligram of C-16 is appreciably the same in all experiments: it depends neither on hemoglobin concentration nor on C-16 concentration (Pepsin concentration is 20 μg/ml).

Concentrations C-16 (mg/ml)	Hemoglobin (mg/ml)	Effective substrate (mg/ml)	Protected substrate per mg of C-16
0, 4	5	4.1	2.25
	10	9.1	2.25
	20	19.1	2.25
0, 8	5	3.0	2.50
	10	8.1	2.38
	20	18.7	1.63
1, 2	5	2.3	2.25
	10	7.3	2.25
1, 6	5	1.5	2.19
	10	6.5	2.19
	20	16.1	2.44
2, 0	5	0.8	2.10
	10	5.3	2.35
	20	15.1	2.45
2, 8	10	4.0	2.14
	20	13.3	2.39
4, 0	10	1.8	2.08
	20	9.7	2.58
Mean values	5		2.26
	10		2.24
	20		2.29

the reaction medium. In the case of the Haemoglobin N.B.C. substrate and the C-16 polysaccharide that we used, one may assume that 1 mg of inhibitor "protects" about 2.26 mg of Haemoglobin.

These results seem to confirm those of *Anderson* [2], *Piper and Fenton* [7, 16], *Ravin, Baldinus and Mazur* [17], which conclude that pepsin is inhibited by formation of a complex between a substrate and the polysaccharide inhibitor. The relative inhibition depends in fact on the concentration of the substrate and not on the concentration of pepsin *(Anderson and Piper):* the formation of a complex between sulphated polysaccharide and substrate is proved by a reduction in metachromasia due to toluidine blue *(Anderson)* or by precipitation of the complex in certain ranges of pH *(Ravin).*

In two previous publications [5, 13] we have demonstrated the formation of a complex between protein substrates and various sulphated polysaccharides. On paper electrophoresis [13] this complex manifests itself by the appearance of a supplementary band, migration of which towards the anode is intermediate between that of the substrate and that of the polysaccharide. During diffusion on agar [5] the complex appears in the form of an arc of precipitation. In both cases it has not been possible to demonstrate the formation of a complex between pepsin and the sulphated polysaccharides.

The only discordant data are the following: *Levey and Sheinfield* [12], found no inhibition if the sulphated polysaccharides are incubated with a protein substrate before addition of pepsin, which is in contradiction with our results. *Accary, Dubrasquet, Agneray and Bonfils* [1], observed the formation at pH 1.6 of a complex between C-16 polysaccharide and pepsin; this complex remains stable on electrophoresis at various pH.

The exact mode of formation of the complex between the sulphated polysaccharide and the protein substrate is still little known, as also are the reasons for the resistance of this complex to peptic proteolysis. One may suppose that the complex results from interaction between the negative charges of the sulphated polysaccharide and the positive charges of the proteins. The polysaccharide might thus mask on the surface of the substrate molecule the sites able to fix the enzyme molecule.

One may finally note that the average weight ratio between the protected substrate and the polysaccharide inhibitor is exactly the ratio of the molecular weights of Haemoglobin (68.000) and C-16 polysaccharide, if one attributes to the latter a molecular weight of 30.000 compatible with the data obtained by light-scattering [4]. It is thus possible that the reaction between sulphated polysaccharide and substrate may be stoechiometric. As one molecule of C-16 con-

tains about 100 sulphate groups, the latter would not intervene in the quantity of substrate linked to polysaccharide, only in the binding force between the two elements of the complex.

Summary

Inhibition of peptic proteolysis by a sulphated polysaccharide, degraded Carrageenan, has been studied by the pH-stat technique. Relative inhibition is inversely proportional to the concentration of the substrate.

By varying the concentration of the substrate and the inhibitor it is possible to show that the inhibitor acts by protecting part of the substrate. The quantity of substrate protected by a given weight of inhibitor is independent of the concentration of the substrate and of the inhibitor.

The mechanism of inhibition of peptic proteolysis by sulphated polysaccharides thus involves the formation of a complex between sulphated polysaccharide and protein substrate.

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Isolation and Detection of Gums in Frozen Desserts*

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The proposed promulgation of definitions and standards of identity for frozen desserts (1) dictates that increased attention be given to the detection and determination of gums in frozen desserts. This topic has been under consideration by this Association for 25 years. Cross (2) reported that there were no suitable methods at that time (1934) and undertook a study of gums in various products, including ice cream. The first report to the Association of a method that showed promise was made by Hart (3) who reported some success with a method for gums in cheese. He detected the presence of gums in authentic ice creams containing locust bean and tragacanth. This method was not studied collaboratively.

Official methods are available for the isolation and detection of gums in soft curd cheese, and in mayonnaise and French dressing (4, 5). With these methods the presence of gums has been detected by reactions of hydrolysis products after the gum has been isolated. Essentially, this isolation technique consisted in removal of fat with ether, precipitation of proteins, and final precipitation of the gum from aqueous solution with alcohol. Experiments conducted by previous Associate Referee showed the procedure would work on ice cream, but results of collaboration were poor. Failures to recover the gum material were frequent, and losses probably occurred when emulsions formed during fat extraction (6). Johnson (7) reported a method for isolating gums in cheese spread in which the problem of emulsions was overcome by using dioxane, which has the property of removing fat and water. This method had not been subjected to collaborative study. Using dioxane to remove butterfat from a laboratory ice cream worked exceptionally well when tried by the Associate Referee.

Chemical methods for identification of gums have been published (8, 9). Newburger, *et al.* (10, 11) published a technique for obtaining the infrared spectra of water-soluble gums. The latter method was chosen for study because of the specificity offered by the technique. A thin film of the gum is prepared and the infrared spectrum, in air, is determined.

Essentially, the method of isolation involves removal of fat with dioxane, precipitation of protein, and isolation and purification of the gum by alcohol precipitation. Obviously the gum must be rather pure

* Presented as the report of the Associate Referee at the Seventy-third Annual Meeting of the Association of Official Agricultural Chemists, Oct. 12-14, 1959, at Washington, D.C.

for identification and, for this reason, the method employs a triple precipitation as cleanup. Dilute HCl (1 + 1) is used instead of acetic acid (4, 5) because carbonyl-type absorption is characteristic of gums. "Salting out" is accomplished with saturated NaCl solution instead of $KAl(SO_4)_2$ solution because NaCl is transparent in the infrared while ionic sulfate shows absorption in the 9 μ region. In all probability these interferences would be present in small amounts; however, time did not permit a study of the effects that might be produced in the spectra.

METHOD

Reagents

(a) *Dioxane.*—Technical grade is satisfactory. (*Caution:* Vapors are obnoxious and harmful.)

(b) *Trichloroacetic acid solution.* — 50%. Freshly prepared as needed from non-hydrolyzed reagent.

(c) *Organo-silicone compound.*—To prepare non-wettable surface. Desicote ® (Scientific Instruments Division, Beckman Instruments, Inc., Fullerton, Calif.) has been found satisfactory.

Apparatus

(a) *Infrared spectrophotometer.*—Recording, for operation in 2–15 μ region.

(b) *Water-repellent plate.*—Wash 3–4" glass square thoroughly with detergent, rinse, and dry with towel. Dip glass rod in organo-silicone compound and streak adhering liquid across plate. Repeat streaking several times. Rub plate with lens paper to distribute evenly; then rub with clean lens paper to remove excess. Plate is now ready for use. Wash plate with cold H_2O and dry with towel after each use. Plate can be reused as long as it remains nonwettable.

Preparation of Sample

Weigh 50 g sample of frozen dessert into 250 ml centrifuge bottle and heat to 60° in H_2O bath. Add 150 ml dioxane, shake vigorously 2 minutes, and centrifuge 10 minutes at 1800 rpm. Decant and discard supernatant. Add 30 ml ether and shake vigorously to break mass at bottom, using rod if necessary. Decant ether and repeat ether wash once. Heat in H_2O bath to remove residual ether. Add 30 ml 80° H_2O and shake vigorously 2 minutes to dissolve or disperse residue.

Separation of Gum

Add 20 ml 50% trichloroacetic acid and heat to 60° in H_2O bath. Shake 1 minute and centrifuge 10 minutes at 1200 rpm. Decant solution through fast folded paper into second centrifuge bottle, and discard residue.

Fill centrifuge bottle with alcohol, add 1 ml saturated NaCl solution, mix, and let stand until coagulation occurs. If precipitate does not form, gums are absent. (Let opalescent solutions stand overnight to facilitate precipitation. Centrifuge and discard if no precipitate is present.) Centrifuge 10 minutes at 1800 rpm and immediately decant and discard supernatant.

Purify precipitated gum by adding 30 ml 80° H_2O and shake vigorously to dissolve or disperse precipitate. Fill centrifuge bottle with alcohol, add 1 ml saturated NaCl solution and 1 drop HCl (1+1), mix, let precipitate coagulate, centrifuge 10 minutes at 1800 rpm, decant, and discard supernatant. Repeat purification step twice.

Chemical Detection of Gum

(a) *Before infrared identification.*—Scrape small amount of precipitated gum from centrifuge bottle with spatula and transfer to 50 ml beaker with about 10 ml hot H_2O. Add 2 ml HCl and boil 5 minutes. Neutralize to multirange indicator paper, using 30% NaOH solution first and completing with 10% NaOH solution. Remove paper and continue as in 15.147, beginning ". . . add 5 ml Benedict's solution."

(b) *Testing large amount of precipitate.*—If infrared spectrum is not desired, spectrum indicates presence of 2 or more gums, or precipitated gum will not form film (as with karaya), proceed as in 15.147, beginning "Add to residue in tube . . ."

(c) *Infrared identification.*—(May not be applicable to mixtures of gums.) Dissolve or disperse residue in 35 ml H_2O. Prepare film as below, obtain infrared spectrum against air, and compare with spectra of reference gums.

Preparation of Gum Films

Place H_2O repellent glass plate over 2" opening on steam bath. Pour enough of aqueous gum solution on plate to form circle about 2" diameter. (Volume required to produce film of sufficient area to block sample beam and of suitable thickness to produce characteristic spectrum varies with nature of

gum and its concentration.) Heat plate until film is dry and remove film with forceps. If film sticks to plate, remove with razor blade or tissue lifter. Transfer film to beaker and dry at 100° for 15–30 minutes. (Excessive heating may char some gums.) Place piece of film between 2 salt plates and obtain infrared spectrum against air.

Preparation of Reference Gum Films

Disperse 0.2 g gum in 30 ml 80° H_2O and add 20 ml 50% trichloroacetic acid solution. Continue as in *Separation of Gum,* beginning "Shake 1 minute and centrifuge 10 minutes at 1200 rpm."

Collaborative Study

Each collaborator was sent seven bottles of dry mix, two tubes of butterfat, and seven reference gums (guar flour gum, locust bean gum, Irish moss, pectin, gum arabic, agar, and sodium carboxymethylcellulose). The dry mix contained 5.7 g nonfat dry milk solids, 6.0 g sucrose, and 1.5 g dextrose. Five of the bottles also contained 0.2 g of a gum equivalent to 0.4% gum in the collaborative ice cream mix. (The proposed standards permit 0.5% gum.) Two of the bottles were marked as practice samples: "With added gum" and "Without gum." The other five bottles were identified by number only. The collaborators were instructed as follows: "Transfer 6 g butterfat and one bottle of dry mix to a 250 ml centrifuge bottle. Wash the bottle with a total of 31 ml hot (80°) water, and transfer washings to the centrifuge bottle. Shake vigorously 2 min. Additional warming and shaking may be necessary to produce uniform emulsions. This will simulate 50 g ice cream. Proceed with analysis, beginning, "Add 150 ml dioxane . . ." [This mixture is within the limits described by Sommer (12)]. Collaborators were not asked to perform the chemical tests for presence of gums.

The procedure for obtaining films of the gum material isolated was editorially changed after the method was sent out for collaboration. Instructions were reworded to prevent ambiguity since one collaborator submitted results that showed he used the entire amount of gum isolated to prepare the films. These films showed complete absorption in some areas; however, the unknowns were correctly identified. Two collaborators had previously reported correct results with excellent spectra before the change was issued.

Results and Discussion

Spectra submitted by collaborators closely parallel those for locust bean, Irish moss, pectin, gum arabic, and sodium carboxymethylcellulose (see Figs. 1 and 2). Results of collaboration are shown in Table 1. Except for Collaborator 8 all collaborators isolated all gums and each identification was correct with the exception of collaborative Sample 3, which contained locust bean gum. Most collaborators stated that locust and

Table 1. Collaborative results for identification of gums in ice cream mix[a]

Sample	Gum Present	Collaborator 1	2	3	4	5	6	7	8	9
With added gum	Sodium carboxymethylcellulose	x	x	x	x	x	x	x	x	x
Without gum	—	x	x	x	x	x	x	x	x	x
No. 1	—	x	x	x	x	x	x	x	—[d]	x
No. 2	Arabic[b]	x	x	x	x	x	x	x	—[d]	x
No. 3	Locust bean[c]	Guar or locust	Guar or locust	Guar	x	x	Guar, possibly locust	x	Locust-guar	Guar
No. 4	Irish moss	x	x	x	x	x	x	x	x	x
No. 5	Pectin	x	x	x	x	x	x	x	x	x

[a] x = Correctly identified. [b] Sometimes called acacia gum. [c] Sometimes called locust seed gum, carob bean gum, or St. John's Bread. [d] See explanation under *Results and Discussion.*

guar gave practically identical spectra. This difficulty was to be expected. Newburger, *et al.* (11) report: "The spectra of locust bean and guar flour gums are indistinguishable. However, some authorities believe that these gums are identical or at most differ in the proportions of substances which they contain." Ewart and Chapman (8) report: "Locust bean and guar gums give identical reactions and cannot be distinguished on the basis of these (chemical precipitation) tests." The Associate Referee found that the two untreated gums had identical spectra, but treating the gums as in the proposed method produced marked differences in the 10 to 10.5 μ region. Guar gum (Fig. 1) showed a minor peak at 10.3 μ; locust bean (Fig. 1) produced a flat plateau between 10 and 10.5 μ. Collaboration disproved this as a means of differentiation. Some collaborators obtained films for locust bean that also showed a minor peak at 10.3 μ.

The samples marked "Without gum" and "No. 1" were identical. Collaborator 8 reported, "powdery precipitate formed which would not give a film" for Sample 1. It is presumed that this material was protein. Possibly not enough trichloroacetic acid was added or the temperature might have been too low to effect good coagulation of the protein. A gum was isolated by Collaborator 8 for Sample 2 and the spectra were submitted; however, no identification was made. He reports: ". . . films for the reference gums were satisfactory except in the case of acacia. The film could not be removed from the glass plate. The film chipped off in fine flakes and could not be used. This was also true of collaborative Sample 1. The film from collaborative Sample 2 was also difficult to handle."

Since this collaborator did not place his films between salt plates, but rather used a cardboard holder with windows, it is presumed this is the reason he did not submit a spectrum of reference acacia. The spectrum he submitted for collaborative Sample 2 could be superimposed on spectra submitted for Sample 2 by other collaborators who used the same model instrument as Collaborator 8.

One collaborator reported Sample 4 contained agar when it actually contained Irish moss. His reference spectra and the spectrum obtained from Sample 4 were given to an analyst in this laboratory who was unfamiliar with the collaborative study or the incorrect identification. This analyst correctly identified the spectrum of Sample 4 as Irish moss. For this reason the reference spectra and spectra obtained from Samples 3 and 4 were returned to the collaborator for re-evaluation. His original identification of Sample 3 as locust bean gum was correct. He subsequently identified Sample 3 as guar gum and Sample 4 as Irish moss. He reported choosing Irish moss on the basis of the spectral region between 10 and 12 μ. This was the same area used by the analyst in this laboratory to distinguish between agar and Irish moss.

The spectra obtained from films of untreated guar, locust bean, pectin, agar, and tragacanth gums (Figs. 1 and 2) do not differ materially from the spectra obtained from films of the gums recovered in the isolation and reference procedures. The only differences were in the relative heights of absorption peaks. Possibly this can be explained by the fact that gums are natural products and contain many compounds. Slight changes in technique between analysts may isolate more or less of a particular compound, giving rise to the differences in relative heights of absorption peaks. Gum arabic (Fig. 2) and psyllium seed gum (Fig. 2) produced essentially the same spectra for treated and untreated material except for slight changes in carbonyl-type absorption in the 6 μ region. Sodium carboxymethylcellulose (Fig. 2) varied considerably in the 5.5 to 8.5 μ region for the treated and untreated gums. The dotted areas indicate the trace for the untreated gums in Fig. 2 (arabic, sodium carboxymethylcellulose, and psyllium seed).

The collaborators were asked to report what type of device was used to hold the films in obtaining the spectra. Two used the conventional liquid cell holder (demountable type), and one a holder normally used in the mull techniques. Collaborator 8 placed his films between two pieces of stiff paper with a window cut to allow passage of the

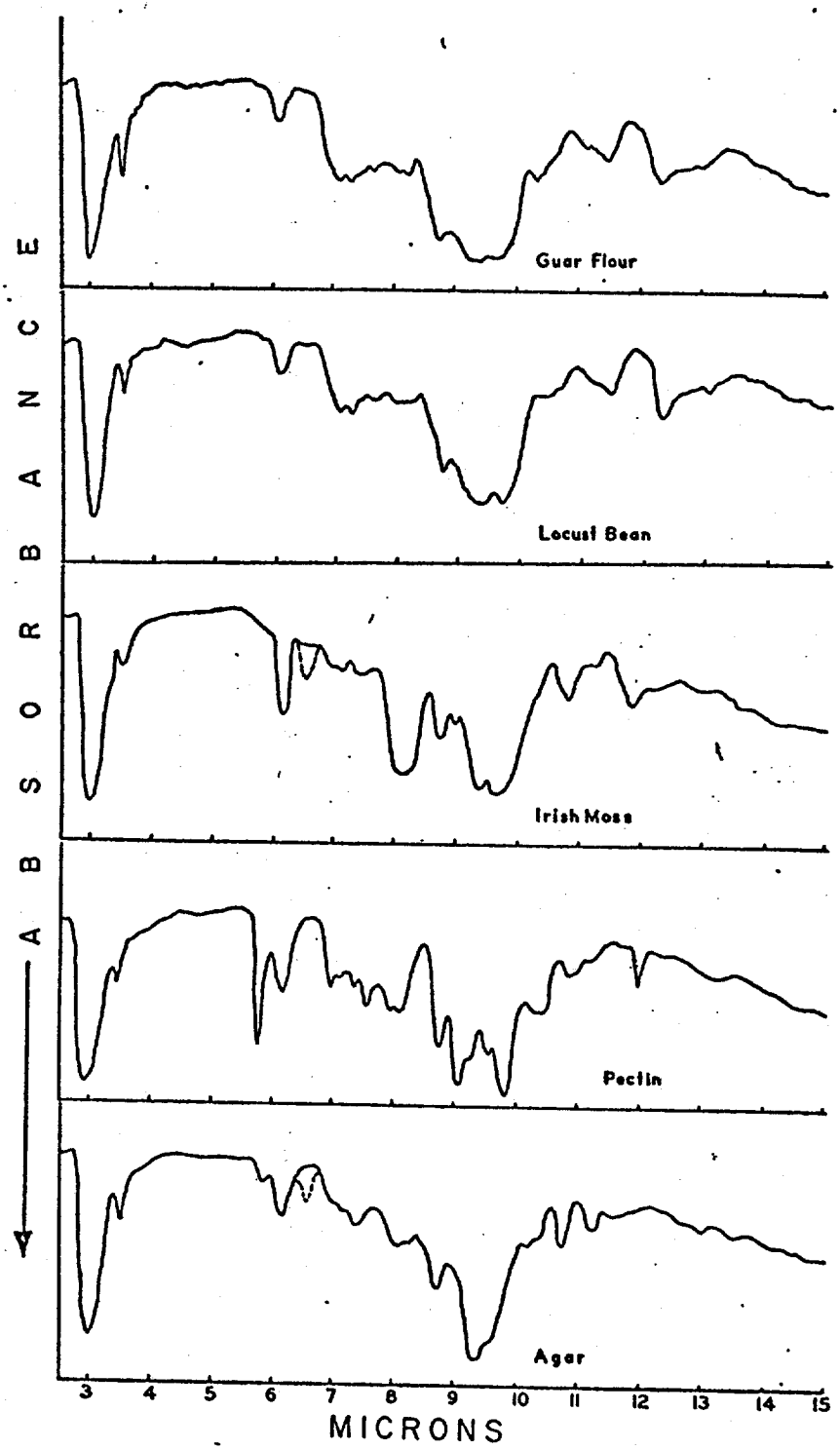

Fig. 1—Spectra of gums isolated by the method.

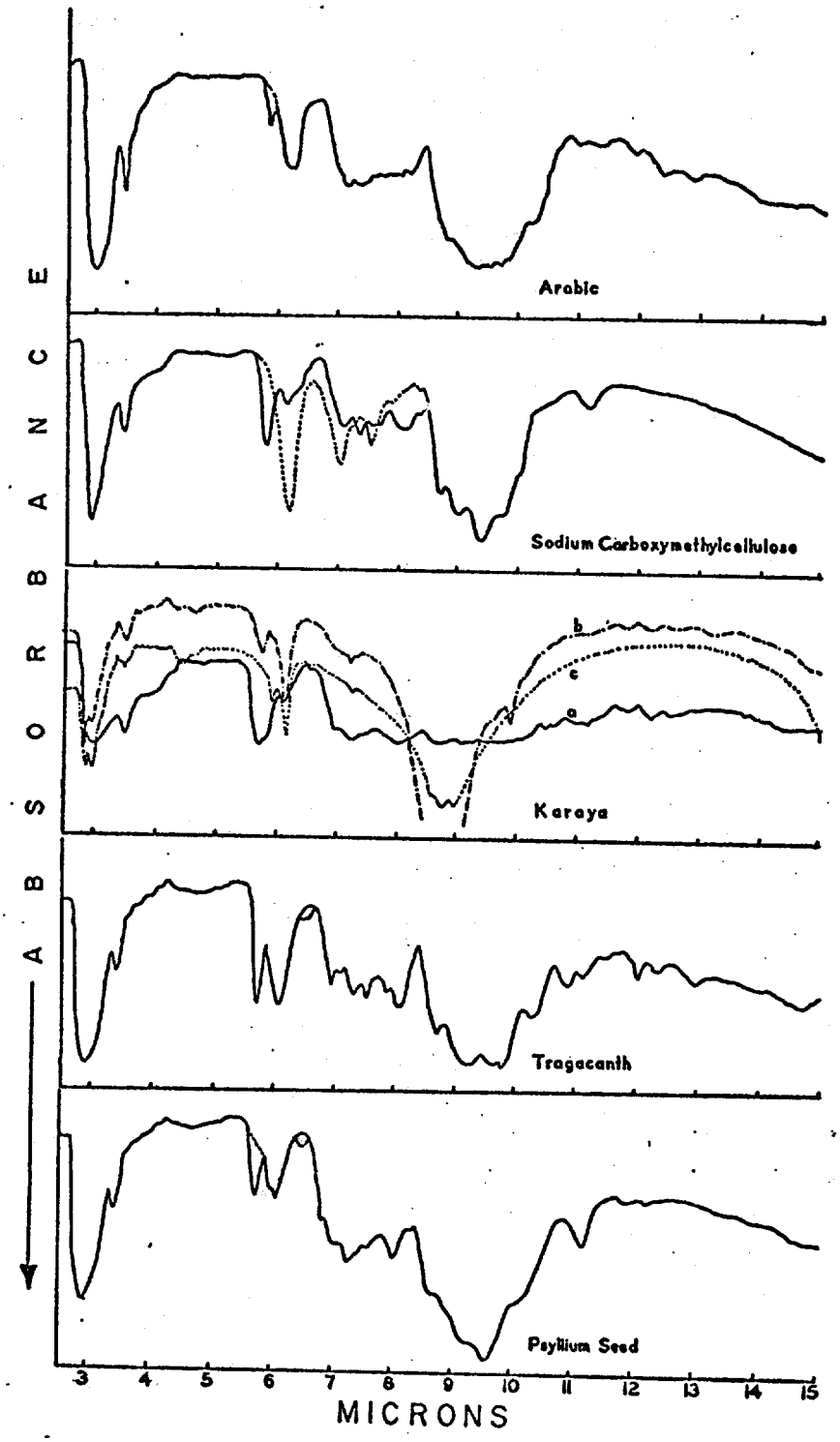

Fig. 2—Spectra of gums isolated by the method.

beam. While this technique is successful with well-formed films it cannot be used with the brittle, flake-type film such as gum arabic produces. The other collaborators used a device similar to the one described by the Associate Referee in his instructions. This device holds a piece of film between two salt plates with tension supplied by rubber bands. It is constructed of pressed board and eye screws. Samples can be changed quickly but it tends to damage portions of the film not covered by the salt plates. If no tension is put on the salt plates, films may fall out of the holder. Pressure may fracture brittle films, cause multiple reflections, and thereby give "jitters" to the recorder pen. While undesirable, this type of spectra does not negate identification since gums are natural products containing many compounds and, because of this, the absorption peaks are broad and an even pen line is not necessary for differentiating between spectra.

Experimental Work

A minor absorption peak at 6.5 μ occurred in each study of Sample 4, Irish moss, which did not appear in the spectrum of the reference gum. This absorption also occurs to a much smaller degree in agar, tragacanth, and psyllium seed gums isolated from the collaborative ice cream mix. (See dashed areas of Figs. 1 and 2). In order to study the nature of this peak the Associate Referee added Irish moss to butterfat, to sucrose, to dextrose, and to nonfat dry milk solids. The peak at 6.5 μ occurred only in the film of Irish moss isolated from the milk solids. A second protein precipitation did not remove this peak and produced an extremely poor, brittle film. The spectrum of isolated Irish moss that had been added to pure casein showed a minor absorption at 6.5 μ but not as intense a one as that from the milk solids. Conditions in this experiment were not exactly the same, however, since the casein was in large granules. It could not be reduced by grinding and, therefore, had to be dissolved in a 1% NH_4OH solution with the Irish moss. Irish moss was isolated from two other commercial brands of "instant" nonfat dry milk and these spectra also showed absorption at 6.5 μ. This type of study has not been conducted on agar, tragacanth, or psyllium seed gum.

A commercial "ice milk" frozen dessert was analyzed by the proposed method. The material isolated formed a film and the infrared spectrum was obtained (Fig. 3a). This spectrum was quite similar to that of tragacanth in the 8.5 to 11.5 μ region. From 11.5 to 12.5 μ the spectrum resembles guar or locust bean. The doublet effect between 5.5 and 6.5 μ occurs with about the same relative intensity as in psyllium seed and tragacanth. None of the other gums studied showed this doublet effect.

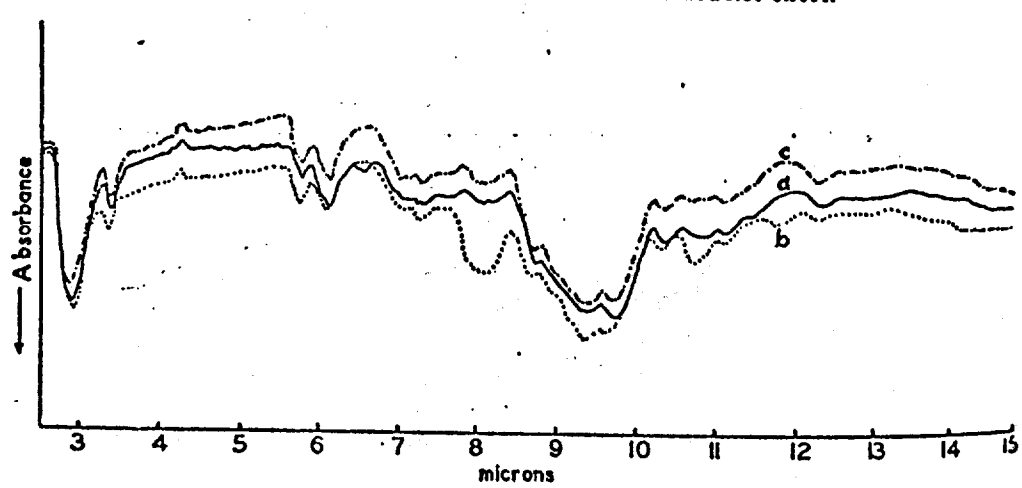

Fig. 3—Spectra of (a) gum material isolated from commercial "ice milk," (b) 1:1:1 locust bean, Irish moss, and sodium carboxymethylcellulose, (c) 1:1 locust bean and sodium carboxymethylcellulose.

The manufacturer of the ice milk said that the gum material used was supposed to contain a 1:1 mixture of locust bean and Irish moss to which he added sodium carboxymethylcellulose in an amount to make the final gum concentration a 1:1:1 mixture of sodium carboxymethylcellulose, locust bean, and Irish moss. A reference spectrum was prepared, using the 1:1:1 ratio (Fig. 3b). This spectrum showed strong absorption at 8 μ due to sulfate absorption in the Irish moss and slight absorption at 11.7 μ—also characteristic of Irish moss. A spectrum was prepared of sodium carboxymethylcellulose and locust bean in 1:1 ratio (Fig. 3c). This spectrum matched that of the gum material isolated from the ice milk. The strong absorptions at 8 μ and 11.7 μ were missing, indicating that no Irish moss was present in the ice milk. A sample of the commercial stabilizer used by this firm was obtained. The gum material was isolated and identified as either guar flour or locust bean gum. Information obtained about the formula for this stabilizing mix indicated it contained only locust bean gum and sucrose.

Chemical tests for the detection of gums were not studied collaboratively. The test with Benedict's solution (15.144(a)) for the detection of gums has been adopted as official (13). This test, in the modified form described in this report, was applied to Irish moss, tragacanth, psyllium seed agar, and the material isolated from the commercial "ice milk" dessert. Karaya isolated from the collaborative ice cream mix was tested by 15.147 (4). All tests were conducted by the Associate Referee and classified positive on the basis of the presence of a yellow, orange, or red precipitate.

The remaining gums permitted by the proposed standards, psyllium seed, guar, oat, tragacanth, karaya, agar, and the alginates were not subjected to collaborative study. The isolation and identification of these gums, with the exception of oat flour gum and the alginates, were studied by the Associate Referee. Each of the gums, except karaya, was isolated and each produced a film suitable for infrared identification (Figs. 1 and 2). Karaya, when isolated from the collaborative ice cream mix, produced a granular precipitate that formed a film with difficulty. The spectrum of this film was obtained (Fig. 2, Karaya b). This material was also ground and the spectrum was obtained by the KBr wafer technique (Fig. 2, Karaya c). These spectra were completely different from the spectra of the reference gum (Fig. 2, Karaya a). Karaya does not form a true solution, and most of the gum is probably lost in the protein precipitation step of the method. Hydrolysis may also be a factor. However, the presence of a gum in a frozen dessert containing karaya can be detected by 15.147 (4). Gelatin, also permitted in the proposed standards, is removed in the protein precipitation. A method for detecting alginates in chocolate products (14), including chocolate ice cream, has been adopted as official (15) by the Association. No oat flour gum was available to the Associate Referee for study.

Summary and Conclusions

A method has been described for the isolation, detection, and identification of gums in frozen desserts. The isolation and infrared identification of five singly occurring gums—locust bean, Irish moss, pectin, arabic, and sodium carboxymethylcellulose—was studied collaboratively with excellent results.

Agar, tragacanth, psyllium seed, and guar gum can also be detected and identified by the method, but they have not undergone collaborative study.

Guar flour gum and locust bean gum cannot be distinguished by this infrared technique. Karaya gum cannot be identified when isolated by the proposed procedure. The presence of a gum in a frozen dessert containing karaya (and/or other gums) can be detected by chemical tests. Brief study indicates that Irish moss, and to a lesser degree agar, psyllium, and tragacanth, binds some material from milk solids that cannot be precipitated by the protein precipitation method described. This material absorbs at 6.5 μ and is quite possibly nitrogenous in nature.

A commercial sample of frozen dessert was studied by the Associate Referee in which he detected the absence of one of the

gums reported to be a constituent of a mixture of three gums.

Recommendations

It is recommended[1]—

(1) That the method of isolation of gums in frozen desserts be adopted as first action.

(2) That the detection of agar, tragacanth, karaya, guar flour, psyllium seed, and oat flour gums in frozen desserts be studied collaboratively.

(3) That methods for separation of mixtures of gums be investigated.

(4) That quantitative methods for gums in frozen desserts be investigated.

Acknowledgments

The Associate Referee wishes to express gratitude to M. J. Gnagy, General Referee on Gums in Foods; and to J. T. Welch, former Associate Referee, for assistance in developing the method; to C. Comstock, Horton and Converse, Los Angeles, who supplied some of the gums used in the study; and to the following collaborators, all of the U.S. Food and Drug Administration: T. E. Byers, E. C. Pranskaitis, P. S. Jorgensen, B. W. Rehn, J. F. Weeks, C. E. Beisel, S. Nesheim, L. F. Schneiderer, A. I. Kleks, and M. J. Gnagy.

[1] These recommendations were approved by the Referee and Subcommittee C and were adopted by the Association. See *This Journal*, 43, 130 (1960).

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FEEDING TEST WITH CARRAGEENIN

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Carrageenin is the hot-water-soluble extractive of the red seaplants *Chondrus crispus* and *Gigartina mamillosa*, and other related species commonly referred to as Irish moss *(5)*. The commercial product contains about 12% moisture and about 80% of the polysaccharide hydrocolloid, which is precipitated in 70% isopropanol. The remaining soluble materials are metallic chlorides and sulfates, and low-molecular-weight organic compounds. About 23% by weight of the hydrocolloid is ash and no fillers are used to standardize the physical characteristics of the commercial product.

The hydrocolloid is now known to be composed of two distinct types of galactosan sulfates (kappa and lambda) in varying but usually equal proportions plus minor quantities of floridean starch, which is a glucosan resembling glycogen. Both kappa and lambda carrageenins consist of sulfated D-galactose units, which apparently must exist in a distinct and definite structural relationship with one another and cannot occur in large separate aggregates *(1, 6)*.

A carrageenin sol is a strongly charged polyelectrolyte with a reported ionization of the sulfates of approximately 60% *(2)*. There is one sulfate unit for almost every hexose unit. Sols of carrageenin are very viscous at low concentration and form thermally reversible gels on addition of specific cations, particularly potassium, ammonium, and calcium.

Because of its valuable colloidal properties and availability in commercial quantities as a refined product of controlled quality, carrageenin has been used extensively in frozen confections; dairy products such as ice cream, chocolate milk and processed cheeses; puddings; bakery and fountain supply specialties; oil emulsions; toothpastes; hand lotions; and various other food, cosmetic, and pharmaceutical preparations. In all of these applications the concentration of carrageenin is less than 1% by weight of the product.

The feeding studies reported here were conducted under a cooperative arrangement with the Seaplant Chemical Company, New Bedford, Massachusetts, in order that the U. S. Bureau of Commercial Fisheries might obtain data on the nutritive value of sea plant products so as to be better able to recommend rational use of this important natural resource.

EXPERIMENTAL

The product used in the feeding studies from 1951 to 1953 was Krim-Ko-gel, High Viscosity (now designated Sea Kem, type 5), which was purchased on the open market except for several lots which were supplied by the manufacturer at the request of the Bureau. The first 2 lots analyzed respectively, 76 and 75% hydrocolloid, 12 and 15% moisture; 25 and 22% mineral matter; by weight. The hydrocolloid content was based on precipitating a 2% solution of the sample with 2 volumes of 99% isopropanol and was the best available index of gum content. The range in viscosity for the 7 lots of carrageenin

used was 180° to 210° MacMichael (2% solution at 40° C., large bob at 20 r.p.m. and no. 26 wire). The gel strength varied from 30 to 60 g. (force in grams transmitted through a 1.1 cm. diameter plunger to rupture a 2% gel of sample aged for 1 hr. in a 25° C. water bath). The arsenic content varied from 0.04 to 10.4 parts per million (Gutzeit test). Since the arsenic content was quite high, a concerted effort was made to look for evidence of toxicity during the conduct of these tests and during the microscopic examination of tissue samples of organs after death of the animals. No histologic evidence of toxicity due to arsenic ingestion was found.

Male and female mice and rats of 2 strains each were allotted at weaning time to groups of 10 animals each. They were housed individually in screen metal cages on wire-mesh floors. The animal room was maintained at 80° F.

The diet used was "Feed, Dry, for Laboratory Animals" (proposed Federal Specification, May 24, 1949, General Services Administration, Washington 25, D. C.) made up of natural feed ingredients. The protein was furnished by dried skim milk, fish meal, animal liver meal, meat scrap, soybean oil meal, wheat, barley, oat groats, corn and alfalfa meal. Supplementary vitamin sources were fish liver oil, wheat germ meal, dried brewer's yeast and dried whey fermentation solubles. This diet contained not less than 23% crude protein and 5% crude fat, and not more than 4% crude fiber and 7% mineral matter. The carrageenin was mixed into the diet at 1, 5 and 15% levels at the expense of equal weights of ground yellow corn, and at the 25% level for a fixed ratio of ground yellow corn, wheat, and oats. These are manifestly high levels of carrageenin, since it is hardly ever included at levels as high as 1% on a dry-matter basis in commercial products. The rats were weighed once a week at which time also the food and water intake for the week was determined. No intermediate data were gotten for the mice. All rats and mice were kept until death (Table 1) at which time a necropsy was done to determine the presence of

TABLE 1

Survival rate of rats and mice fed different levels of carrageenin

Diet designation		Number on test	Survivors after months of test							
			3	6	9	12	15	18	21	24
Rats:										
Control										
(1951)		10	10	9	9	8	7	7	6	3
Carrageenin:										
(1951)	1%	10	10	9	9	9	7	6	4	0
"	5%	10	10	9	8	6	5	4	3	3
"	15%	10	10	10	10	10	9	9	5	2
"	25%	10	10	10	9	8	8	7	3	2
(1939)	5%	10[1]	10	9	9	9	8	6	5	2
Mice:										
Control										
(1951)		10	9[2]	9	9	7	5	4	1	0
Carrageenin:										
(1951)	1%	10	10	10	9	7	6	1	1	0
"	5%	10	9	9	7	7	6	4	1	1
"	15%	10	8[3]	6	5	5	5	4	2	1
"	25%	10	10	10	8	8	7	6	5	4

[1] Half of the rats had received the 10% level in the diet for the first 10 weeks.
[2] Mouse escaped from cage after 11 weeks.
[3] Mouse killed accidentally after 6 weeks.

gross evidences of disease. Organ sections or entire organs of each animal not too badly decomposed were preserved in buffered formaldehyde solution. Later, these were sectioned and examined microscopically, and an evaluation was made of the probable reasons for death.

For comparison, previously unreported data are included for lifetime studies of male rats of the Yokelson strain fed the 5% level of Irish moss gum for which the data for the first 10 weeks have been reported by Nilson and Schaller *(3)*. The diet fed in this instance

consisted of Irish moss gum, lactalbumin, sucrose, dextrin, lard, brewer's yeast, wheat germ, cod liver oil, and salt mixture. The control diet contained about 20% crude protein, 18% crude fat, 43% carbohydrates and 5% mineral matter. Data for the control rats of this series have been reported by Nilson and Wagner *(4)*.

RESULTS AND DISCUSSION

Data in Table 1 indicate that the animals consuming diets containing the different levels of carrageenin lived as long as those eating the control diet without sea plant gum. Female rats and mice generally lived longer than did males, but there is no evidence that diet was a factor. It is concluded that the diets consumed were apparently not a factor in determining length of life.

Only the rats consuming the diet containing the 25% level had lesions suggesting hepatic cirrhosis, which could indicate some dietary deficiency or insufficiency. The incidence was not particularly high, and so the deficiency related to the above was probably marginal. Respiratory infections were the chief cause of death of the rats in all groups. There were lesions of many origins present, but no single type of lesion was consistent in the rats of all groups. Rats consuming diets containing less than the 25% level of carrageenin showed no evidence of injury to parenchymatous organs directly or indirectly. These conclusions, based on gross observations on necropsy, also applied to the rats of the 1939 series.

There was no evidence of damage due to nutritional deficiency in the mice. The studies indicated that respiratory infection, suppuration, and metastatic abscesses were frequently responsible for death. Malignancy was fairly common in all groups, including mammary carcinoma, leukemia, and lung tumors.

In summary, most of the rats and mice suffered from extensive pulmonary infections with metastasis. There were no evidences of lesions of nutritional origin except for the cirrhosis found in the group of rats eating the diet containing 25% carrageenin. Except possibly in this instance, there was no evidence of toxic, corrosive, or other deleterious effects of the diets on the gastro-intestinal tract, kidneys, or livers. The data for these species indicate that carrageenin is a wholesome product except possibly at levels in excess of about 20 times the highest level used in foods. Also, these foods normally supply only a small fraction of the total food intake of the consuming public.

A study of utilization of carrageenin by the rat would be useful, since the data on length of life and reasons for death did not indicate that the diets were implicated to any significant extent. Data in Table 2 show that only with rats consuming the diet containing the highest level fed (25% carrageenin) was the mean maximum live weight reduced. These rats averaged about 90% of the mean maximum weight of the negative control group, and their mean weight was barely statistically significantly different at the 5% level from the mean maximum weight of the rats fed the 5% level of carrageenin, namely, 252 and 296 g. respectively. This decrease in size was apparently due to lowered intake of essential nutrients. There was no indication that the other differences in mean maximum weight were due to unwholesomeness of any diet ingredients, but was determined by the different numbers of the 2 sexes in the groups, the earlier mortalities due to non-specific reasons and strain of rats.

TABLE 2

Data on feeding tests with carrageenin conducted from weaning to death of the rats

	Diet designation					
	Control	Level of carrageenin, %				
		1	5	15	25	5
	(1951)	(1951)	(1951)	(1951)	(1951)	(1939)
Number of rats	10	10	10	10	10	10
Mean maximum weight, g.	289	334	296	305	252	610 [1]
Coefficient of variation, %	30	34	23	25	20	25
Mean weekly basal diet intake, g.	90	101	103	117	118	98
Coefficient of variation, %	20	26	17	18	11	17
Mean weekly water intake, g.	222	250	265	313	348	197
Coefficient of variation, %	10	24	18	21	16	18
Mean apparent digestibility of carrageenin during tenth week, %		64	51	41	46	52
Coefficient of variation, %		39	35	24	28	29

[1] Male rats only of the large growing Yokelson strain.

Data for mean food intake indicate that the rats consumed more food, from 101 to 118 g. weekly, as the level of carrageenin increased, in order to try to ingest maximum nutrients. Mean weekly intakes of carrageenin during the 1951 experiment were 1, 5, 18, and 30 g., respectively. The increased mean weekly water intake was 28, 8, 5, and 4 g. per g. of carrageenin ingested as the level in the diet increased from 1 to 25%. This decrease of water intake per gram of carrageenin was probably determined by the physical limit in the volume of food that could be handled by the gastro-intestinal tract.

Studies indicated that the mean apparent digestibility by rats of carrageenin during the tenth week of the experiment was roughly 50% irrespective of the level fed. It cannot be determined from these studies how much was actually used by the animals and how much was used by bacteria. The finding that the rats fed the diet containing the 25% level of carrageenin may have suffered nutritional deficiency or insufficiency is not surprising, since the bulk handled was large and the feces were often very loose, suggesting that mechanical loss of nutrients could not be discounted. The surprising finding is that these animals, as well as those receiving the 15% level, could handle such volumes of hydrated gums with so little apparent discomfort. Only one rat, at necropsy showed any evidence of impaction of intestines, and it consumed the diet containing the 15% level of carrageenin. In commercial practice the carrageenin is hydrated and used in such small quantities that a high consumption rate by humans is not a problem.

The mice were fed free-choice the same diets and water as fed to the rats. Food or water intake data were not kept. In general, all mice seemed to be able to consume enough nutrients to maintain well-being and there was no trouble with impaction, dermatitis, or excessively soft feces.

CONCLUSIONS

The data indicate that carrageenin is free from any deleterious substances which adversely affect the health of rats and mice or utilization of nutrients, except possibly for mechanical interference when excessively high levels are fed.

Acknowledgment

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A double helix structure in food

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A double helix structure in food

Jellies consist of up to 99 per cent water yet they can wobble without falling apart. The high stability of one jelly, derived from seaweed, is a result of the close bonding of thread-like polymers in the form of a double helix

JELLIES CONSIST largely of water and yet are stable and retain the shape given to them by a mould. The presence of a small percentage—between one and five per cent—of a gelling agent is enough to capture a mass of water and give it quite different properties. Studies of the molecular structure of thread-like molecules of gelling agents are bringing new understanding of food textures and a beginning is also being made in understanding similar systems in living tissue.

Though strictly not foodstuff, certain seaweeds have a layer of jelly that acts as padding to protect the cells from damage by stones that are rolled around by the tide on the beach; the jelly also serves to keep the cells in wet, salty surroundings at low tide. One of these jellies, known as carrageenan (or more strictly *ι*-carrageenan) can be extracted for commercial use and has properties which point to future developments in food technology.

Carrageenan is a poly-saccharide which has sugars as the fundamental building units. Sugars are themselves quite complicated molecules, with ring structures made up from carbon, oxygen and hydrogen atoms. In polysaccharides, the sugar rings are joined to form long chains, or threads, such as those of cellulose or they may sometimes be combined further in branched structures as in starch—the major source of energy in human food. They are very common in nature; indeed, cellulose is the main structural support material in plants and is fundamental to the properties of timber, cotton textiles and paper. It is nature's most abundant organic chemical and therefore the most plentiful of the raw materials which can be renewed, since about 10^{11} tonnes are made each year by natural processes.

Like cellulose, carrageenan contains long chains of sugar units, but these units are of two types (known as *β*-*D*-galactose 4-sulphate and 3,6-anhydro-*α*-*D*-galactose) arranged alternately and with appendages in the form of sulphuric acid combined as an ester. Carrageenan dissolves in warm water without difficulty but the solution can set to a stiff gel which keeps its shape when tipped out of the container and behaves, in many ways, as a solid rather than a liquid. Such behaviour is familiar in gelatin, the base for domestic fruit jellies, and agar (agarose) which is also a polysaccharide, related to carrageenan, with many uses in biology and biochemistry because of its gel properties. A gel made from one part of carrageenan and 99 parts of water has properties which are very different indeed from 100 per cent water. The gel does not flow like pure water and the setting and melting temperature may be 50°C above the melting point of ice.

How can the presence of so little carrageenan alter the properties of so much water? Why does the jelly wobble rather than flow? This is a question which first began to fascinate me about 10 years ago and now it is possible to begin to give some answers.

THE CHEMICAL FORMULA of carrageenan was first determined in our laboratory by breaking the molecule into fragments which could be separated and identified. The intact structure was then deduced as a 'mental reconstruction', in much the same way that one would solve a jigsaw puzzle except that the rules of chemistry were used to decide how the pieces must fit together.

Although this method led to the formula, it does not enable a complete specification of the shape. The shapes of the sugar rings can be predicted from an understanding of the interactions within them; but, even with these parts fixed, the chain is flexible because of twisting about the bonds between units.

To discover more about the shape of the chain, we used X-ray diffraction. More than any other technique, this has made possible the discovery in recent years of the detailed shapes of DNA and proteins. When a crystal is put in an X-ray beam the rays are scattered in a regular pattern which is determined by

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the structure of the molecule and the way it packs with other molecules in the specimen. The intensities of the scattered rays in the pattern can then be measured and used to reconstruct an image of the structure by mathematical methods. This process is often difficult and requires great skill and imagination, but it is worth the effort because it leads to an image which can be very detailed indeed for crystals in which the molecules are packed regularly in three dimensions.

The carrageenans do not form crystals, but only oriented fibres in which the long threads lie side by side and the contacts between them do not conform to a perfectly neat recurring pattern. This irregular packing has the effect of blurring the scattered X-rays so that they do not give evidence which is detailed enough to reconstruct the molecular structure in a detailed way. Fortunately there is an alternative approach to the interpretation. If there is a periodicity in the shape of the stretched out thread in the specimen, this causes a periodicity in the pattern of scattered X-rays. We were able to make observations of this kind and consequently to deduce that the chain repeats periodically every 260 nm (one nanometre equals 10^{-9} metre). Secondly, a helical chain can usually be recognized from the pattern of diffracted X-rays and the number of distinct segments in each turn of the helix can be deduced. In the case of fibrous carrageenan the thread was found to be helical with three segments in each turn. Since the composition of each segment was already known from chemical evidence, the outline of the structure of the whole molecule could then be visualized, but a small inconsistency remained. The density of the fibrous specimens was measured and found to be twice the value expected from the shape and composition of the helix. Therefore, we had to consider the possibility of packing twice as many atoms into the same space. A neat way of resolving this problem is to put two threads together in a double helix.

The thread could probably be draped in many ways along the helical contour, and our next objective was to compile a list of possibilities. To make sure that the actual shape is included in the list, the search must be exhaustive, and the task is so enormous and intricate that it is only feasible to do it with a computer. The problem is simplified to the extent that each segment in the chain is equivalent—that is to say it lies on the same helical contour—so that we need only consider the shape of one typical segment. Further simplification is possible because the shapes of the sugar rings can be taken for granted.

The task for the computer is then to explore the consequences of varying the four angles of twist shown in the formula. The computer is programmed to proceed stepwise through a complete revolution of each angle for every value of all the others. At each stage, it calculates whether a segment with those particular values for the angles could fit on the helical contour. Some of the segments which emerged from this search with the correct shape could be eliminated by a further calculation which showed that they have pairs of atoms impossibly close, and more still were rejected because they left no room for a second chain to form a double helix. Each angle was actually taken in 36 steps of 10°, with interpolation to ensure that no possibilities were missed between the steps. Thus there were at least 36 by 36 by 36 by 36 or about 1.68 million shapes to consider. Some 40 of these were found to fit on the helix contour without having any pairs of atoms impossibly close, of these only 17 had space for a second strand to form a double helix. However, all except one of these suffered from slight cramping of atoms. The final model, selected from almost 2 million possibilities, was the only really convincing one.

We then built 'ball and spoke' models from co-ordinates produced by the computer and looked for a reason why the double helix should form. One possible source of cohesion would be hydrogen bonding similar to that found in other biological helices such as DNA, RNA and the polypeptide α-helix. In the carrageenans, such hydrogen bonds would have to be formed between an OH grouping and another oxygen atom. As the formula shows, each segment of the helix has only two OH groupings, and we were excited and delighted to find that both were engaged in hydrogen bonding between the strands. No doubt other cohesive forces are also involved, but the existence of the predicted hydrogen bonds makes the double helix a more convincing sort of structure.

Any independent evidence for the hydrogen bonds would add strength to the evidence for the double helix. The space filling molecular models suggest that the hydrogen bonds are buried in the interior of the helix, especially when it is remembered that the layer of cations is not shown and would partly cover the surface. The hydrogen bond would be tucked away and protected from chemical reactions such as the exchange of hydrogen for its deuterium isotope. This exchange reaction can be followed by infrared spectroscopy, which uses the principle that OH groups absorb infrared radiation of a very specific wavelength to excite stretching of the OH bond. It is possible to detect OH groups which are engaged in hydrogen bonds, because the wavelength of their absorption is displaced characteristically. Radiation absorbed by OD groups is at quite a different wavelength.

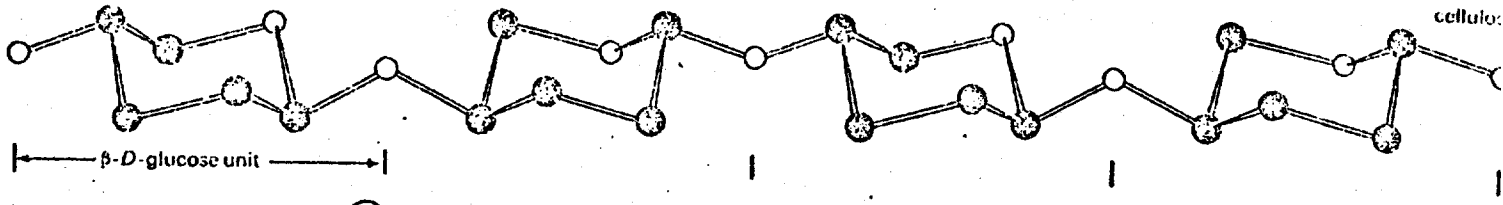
As expected from the double helix model, some OH groups resisted exchange when the sample was exposed to deuterium oxide (D_2O)—better known as 'heavy water'—and these were shown by their wavelength of absorption to be hydrogen bonded. Even more important results were obtained with a sample in which the helices were aligned and the absorption of polarized infrared radiation was measured. This radiation can transfer energy to the sample in one plane only, and the plane can be systematically tilted to locate the direction of OH vibration in the helix. It emerged from these experiments that the hydrogen bonds lie perpendicular to the axis of the helix—exactly as required by the model.

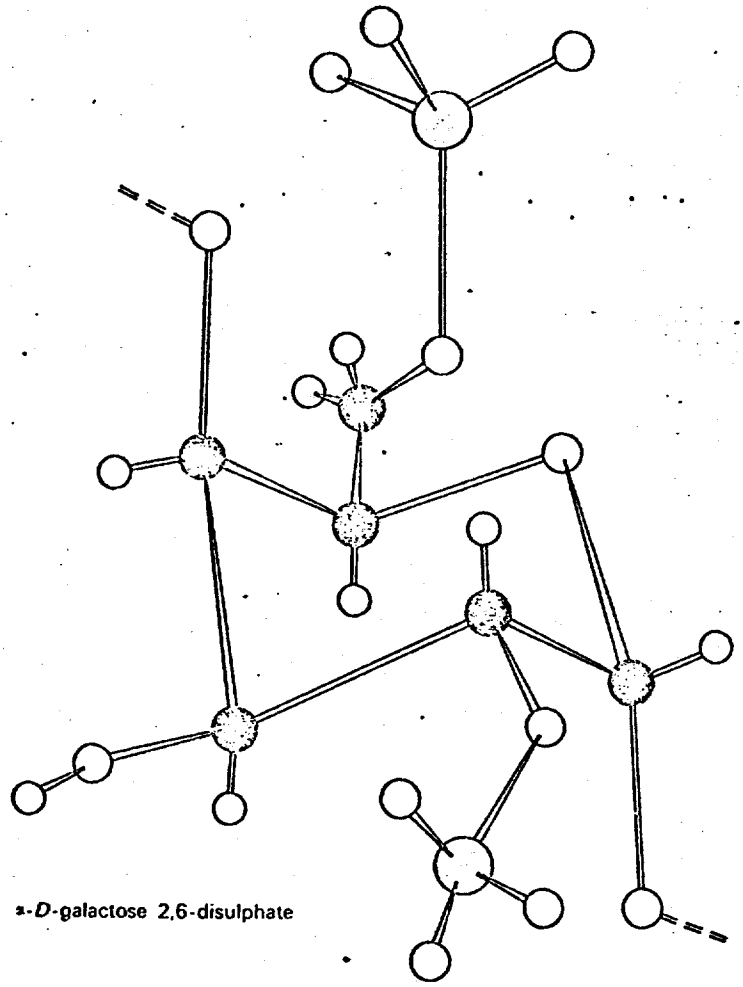
JELLIES DO NOT FLOW and they must therefore have some structure which is perhaps formed by cross linking of polysaccharide molecules in a three dimensional framework. Such cross linking could occur by the formation of a network of double helices. If double helices are present in jellies then they should be detectable as they might increase the optical rotation of polarized light. This is to be expected as each helical chain has a right handed rather than a left handed twist. This 'handedness' represents a dissymmetry in addition to that of the sugar units, which, like almost all other biological blocks have a constitutional dissymmetry of their own.

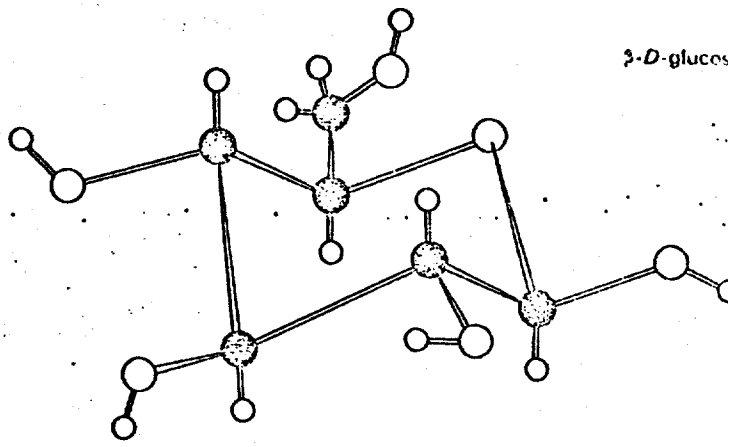
The plane of polarization of light is rotated in one direction or the other depending on the handedness of the molecules in the path. Light is used with a wavelength in or near the visible, rather than the infrared range, because we are interested in the electronic structure of the molecule rather than, as in the preceding section, the motions of atoms or nuclei in bond stretching. When a carrageenan gel sets, there is indeed a sharp change in optical rotation as might be expected if helix formation were proceeding with the extra dissymmetry that it involves.

The extra dissymmetry is brought about by twisting of groups about the four bonds between the sugar units and it was possible to develop a theory to predict the exact optical rotation from the angles in the models. The agreement with experiment is almost perfect and supports the belief that these gels form by the growth of networks of helices.

The mechanism explains many other properties of the melting and setting process. For example, carrageenan gel

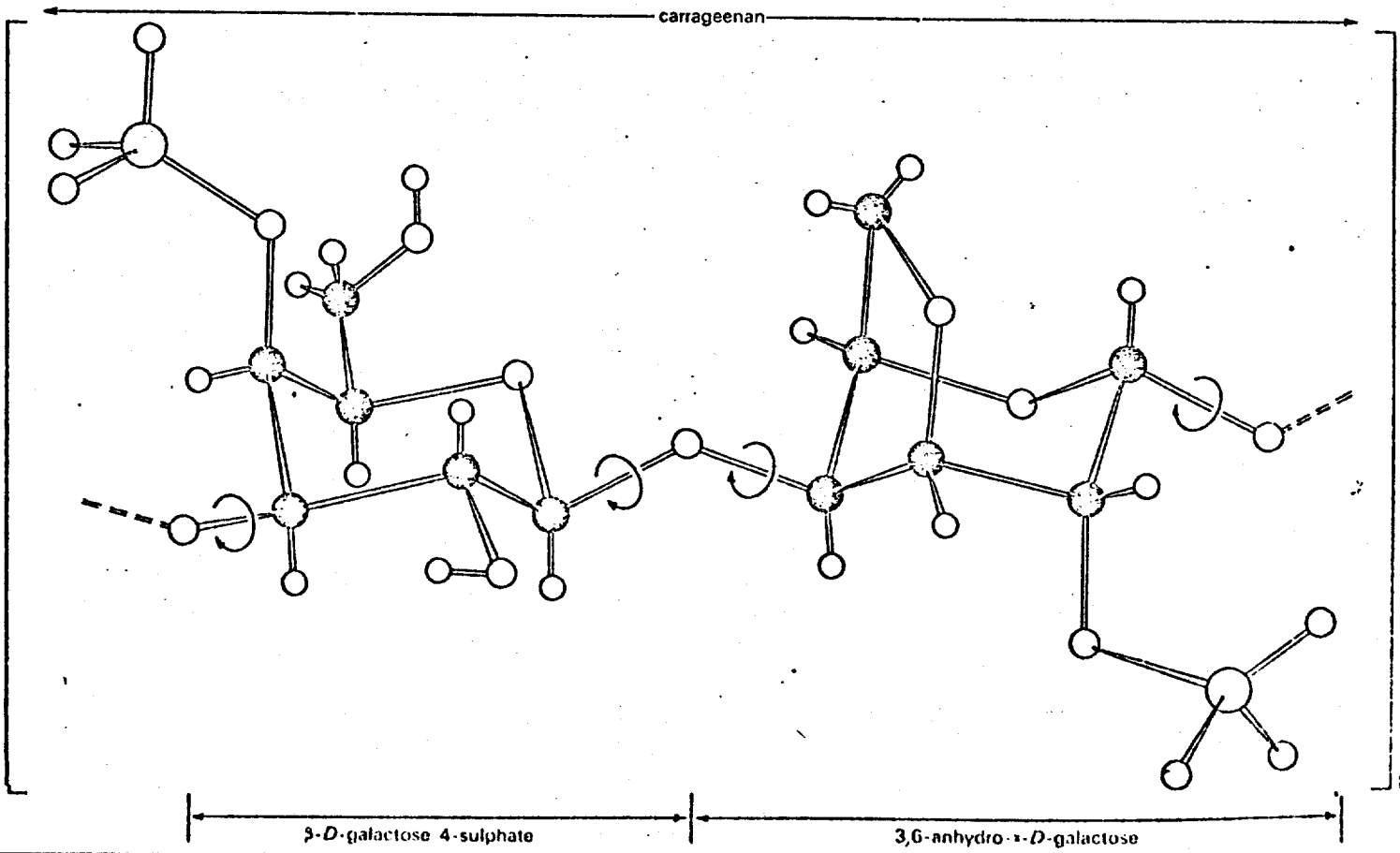


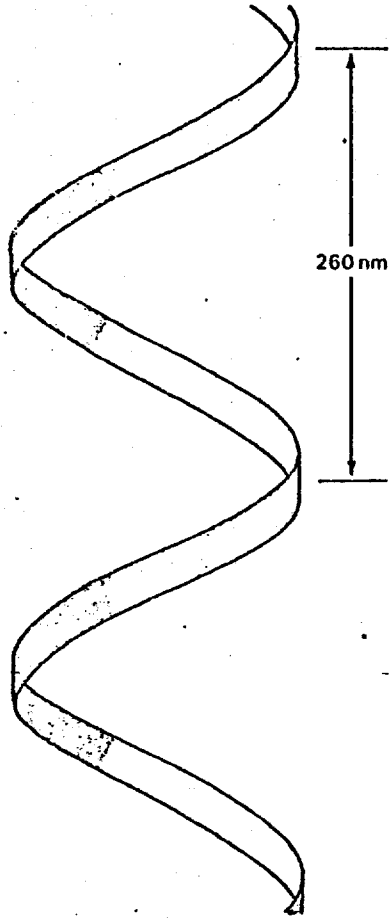


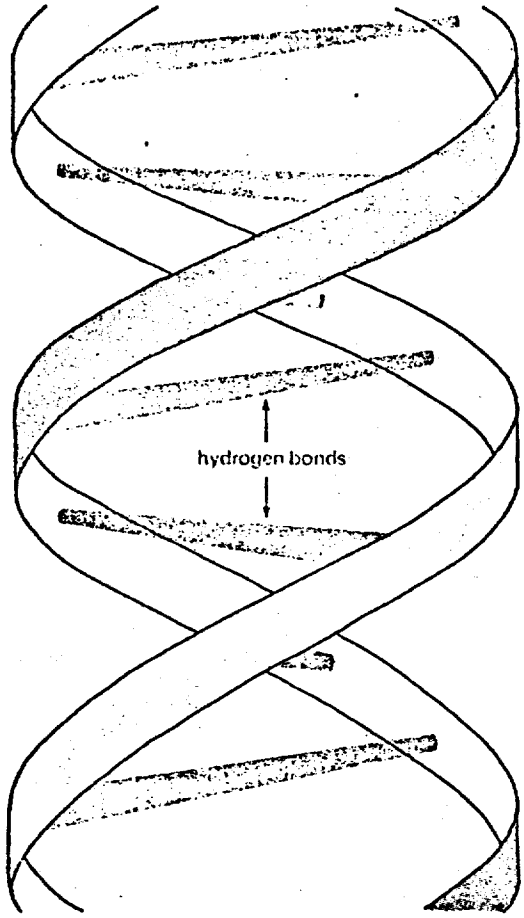


CARRAGEENAN is a polymer similar in construction to cellulose—the basic material of wood and paper—cellulose is a long fibrous molecule built from thousands of glucose units as shown in diagram (top). Carrageenan molecules are built from hundreds of galactose sugar units. These galactose units are of two kinds which alternate in the polymer molecule (bottom). Angles of twist in the molecule which were explored by computer, are shown by arrows. The regular two sugar sequence may be interrupted when 'helix breaking' α-D-galactose 2,6-disulphate (left) introduces a kink into the structure

oxygen atoms | carbon atoms | sulphur atoms

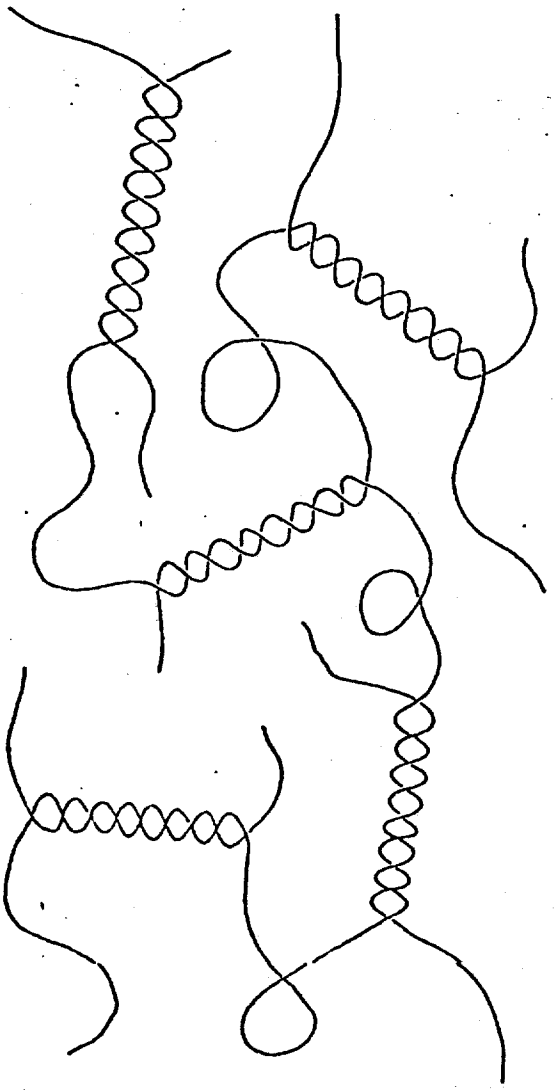


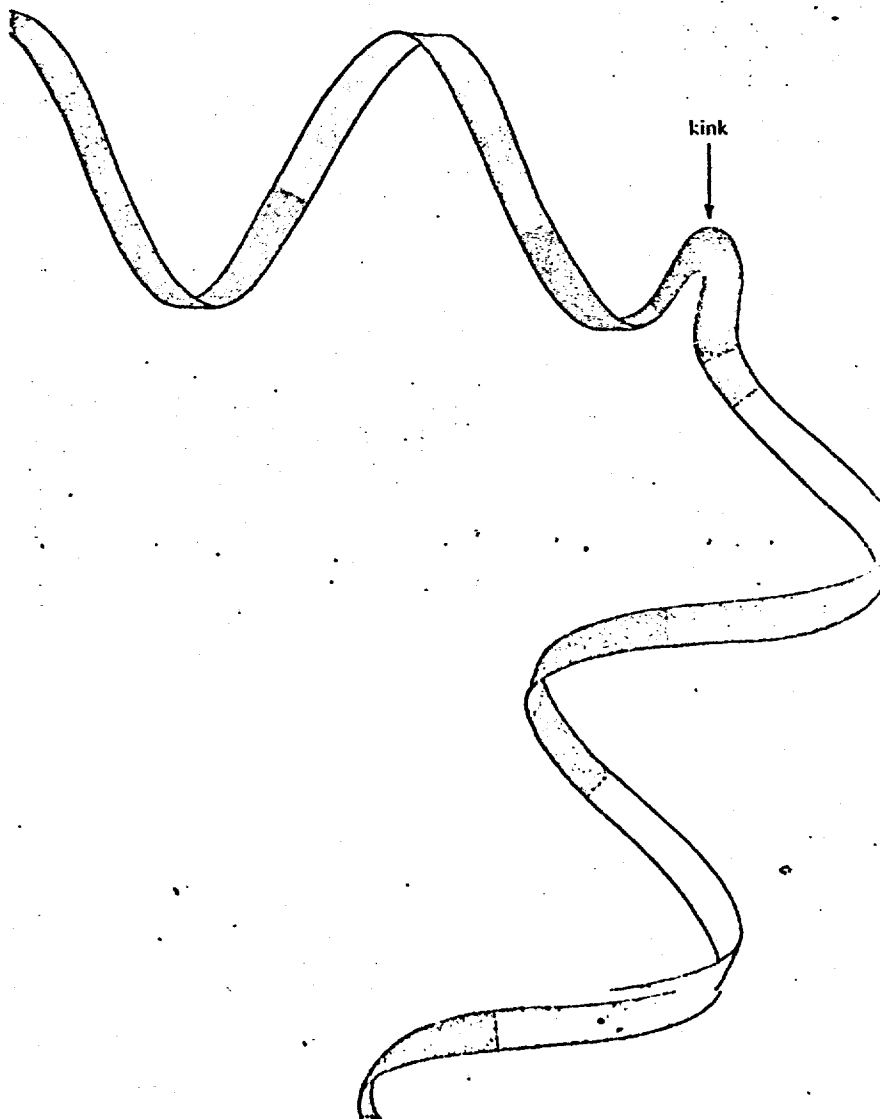


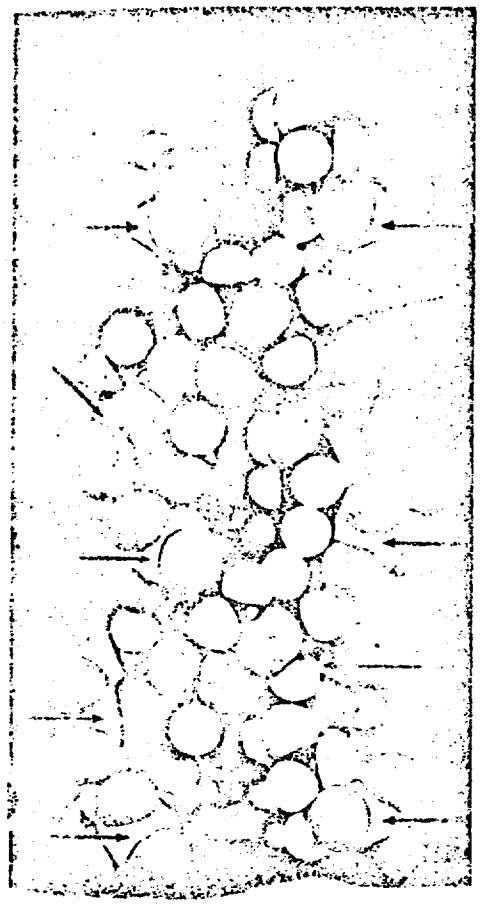


SPACE FILLING MODEL of carrageenan helix (right) shows how the atoms pack tightly together. White atoms are hydrogen, brown are oxygen and black (scarcely visible) carbon; the positions of sulphur atoms are shown by arrows

STRUCTURE of carrageenan gels results from the formation of double helical stretches between different threads (bottom left). Helical stretches are stabilized by hydrogen bonds (left); kinks in the chain are introduced by the presence of α-*D*-galactose 2,6-disulphate molecules (below). The helix consists of three equivalent segments which together comprise one complete turn as shown at far left







set and melt sharply—like the typical crystallization and melting of solids—but the processes in gels are distinct in that the setting and melting temperatures are not always equal. This can be explained if the formation of the double helix is considered to occur, turn by turn, from a pair of chains moving freely in solution. The chains must come together in the correct orientation and register to start forming the helix against opposition from their random motion. When the first turn has been formed it is held together by hydrogen bonds and other forces between the segments in each chain.

Although each segment can in principle form two hydrogen bonds, the geometry of the helix determines that one hydrogen bonding site must be left unsatisfied at the end of each turn. Formation of the second turn of the double helix is much easier because the chains do not have to find each other and are already held in the correct orientation and register. By addition of the second turn, it is also possible to satisfy one of the extra sites for hydrogen bonding. A third turn satisfies the extra site on the second turn, and so on. Therefore, once the first turn of the helix is formed, the remainder forms very easily and suddenly and so the gel sets sharply. Similarly, once the helix starts to break up, the process continues to completion and the gel also melts sharply.

The difference between the melting temperature and setting temperature can be as great as 50°C in some gels of this type—an observation which fits into the theory. The helix melts when it is not quite stable enough to withstand thermal agitation. If, after melting, the temperature was brought slightly below the melting point, the helix as a whole would be stable enough to survive if it could reform, but it cannot because it is specially difficult to form the first turn. Further cooling is required to slow down the motions even more, before it can form. Similar behaviour is known for biological helices of other types, although it is not usually so pronounced as in carrageenan.

THE PROTECTIVE and structural properties of carrageenan in the seaweed would be altered by any change in the extent of cross linkage between molecules—that is, by any change in the number and distribution of double helices. The blend of properties which is most appropriate for the biological needs of seaweed might change from one environment to another. The distribution of helices would therefore be expected to be biologically controlled. There is evidence that this actually happens because, as shown in the formulae, the natural polysaccharide has a proportion of units (α-D-galactose 2,6-disulphate) which interrupt the normal alternating pattern. These units were shown by means of model building in the computer, to cause a kinking which could regulate helix formation. Wherever the carrageenan chain is interrupted by replacement of 3,6 anhydro- α-*D*-galactose by α-*D*-galactose 2,6-disulphate a kink in the chain occurs. More conclusively still, we were able to find a natural enzyme which could be involved in the biological de-kinking of chains to increase helix content as required, and which we have named 'dekinkase'. The porosity, rigidity, strength, cation exchange and water holding properties of carrageenan gels may be biologically altered by means of this enzyme and hence the shape of parts of the seaweed itself may be altered. Investigations are just beginning of general molecular mechanisms for the control of biological shape and the carrageenans are interesting as a simple example of control over shape.

The carrageenan system can probably be regarded as a prototype for the control of properties of polysaccharide networks throughout nature, although the mechanisms are likely to be more subtle and elaborate in more highly organized forms of life: and other forms of linking besides double helices will no doubt be found. The helical network structure is immensely strong enabling a jelly to consist of 99 per cent water and yet to wobble and return stably to its original position. In the plant cell wall, such networks are involved in many functions, including the control of cell enlargement and hence growth. Certain plant hormones loosen the cell walls to permit enlargement by stimulating metabolic processes to act on the network. In connective tissues of animals and humans, the importance of carbohydrate networks is shown by the unpleasant effects to be found when they deteriorate in such diseases as arthritis.

Many staple foods, such as bread, rice, fruit and potatoes are good to eat because of their texture as well as their taste. The creation and preservation of texture is as urgent a problem in food technology as flavour or nutritional value—for example, edible proteins from plant sources and the bacterial conversion of hydrocarbons would be much more useful if they could be given acceptable textures. Polysaccharides are already used for texture in many manufactured foods, from ice cream and milk shakes to potato chips and tinned meat, and they are very important for such natural textures as vegetables and bread.

Although carrageenans are not digested by man and do not act as a source of energy like starch it has many practical uses in the food industry. The simple network of molecules in carrageenan, cross linked as it is by double helices, might be used to give different textures to food depending on the pattern of cross linking and the properties of the helix. Yet more variety can be achieved by using polysaccharide gels of other types. A great deal remains to be discovered about the origin of textures by further study of polysaccharides but there is still an enormous technology gap between what man can achieve in the laboratory and what nature achieves every day.

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FURTHER READING:

THE SHAPES OF MOLECULES: CARBOHYDRATE POLYMERS by D. A. Rees *(Oliver and Boyd, Edinburgh, Contemporary Science Paperback No 14, 1967)*

STRUCTURE, CONFORMATION, MECHANISM IN THE FORMATION OF POLYSACCHARIDE GELS AND NETWORKS by D. A. Rees (in *Advances in Carbohydrate Chemistry and Biochemistry, 24, 1967*)

ACKNOWLEDGEMENTS

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Sulphuric Esters of Polysaccharides as Activators of a Bradykinin-forming System in Plasma

PREVIOUS work[1] has shown that intravenous injection of agar, a naturally occurring sulphuric ester of polygalactose, leads to an appreciable reduction in bradykininogen concentration as well as to a significant increase of *p*-tosylarginine methyl ester (TAMe) esterase activity in the plasma of the rat. Other results indicate that, besides agar, synthetic sulphuric esters of certain polysaccharides are potent activators of the bradykinin forming system in rat or human plasma *in vitro*. In the experiments demonstrating this activity, 0·2 per cent oxalated plasma was collected, avoiding contact with glass, and treated with 1 mg/ml. of 8-hydroxy quinoline (8HQ), an inhibitor of enzymes which destroy bradykinin[2]. Kinin formed was assayed on isolated, atropinized guinea-pig ileum which had been treated with antihistamine, using synthetic bradykinin as the standard. Powdered cellulose, glycogen from shellfish, inulin, soluble starch and agar were added to rat plasma, either as such, or after conversion to water soluble sulphuric esters by treatment with chlorosulphonic acid in pyridine[3]. The effects of carrageenin, a sulphuric ester of polygalactose, extracted from Irish moss, as well as of heparin, were also examined. When incubated at 37° C with rat plasma, all sulphuric ester polysaccharides caused stimulation of smooth muscle equivalent to, on average, 1·0 μg of bradykinin/ml. The active material formed had the characteristics of bradykinin; it was rapidly destroyed by plasma in the absence of 8HQ, as well as by chymotrypsin. Maximum activation was reached 1 min after the addition of 10^{-4} or 10^{-5} g of cellulose sulphate to 1 ml. of plasma (Fig. 1). Larger concentrations (10^{-3} g/ml.) of this activator were less effective during the early phase of the incubation, which suggests an inhibitory effect of excess sulphopolysaccharide on bradykinin production. Ten parts by weight of hexadimethrine bromide, previously shown to inhibit kinin formation[4], inactivated one part of cellulose sulphate. None of the non-sulphated, native polysaccharides induced kinin formation; heparin, a sulphated derivative of polyglucosamine and glucuronic acid, previously shown[5] to be an activator of certain kinin-forming agents, was much less active than the other sulphopolysaccharides examined. The amounts of bradykinin formed by optimal concentrations of cellulose sulphate in rat plasma were the same as those resulting from the exposure of plasma to powdered glass[6]. Either treatment led to a comparable depletion of plasma-kinin precursor, so that pre-incubation of plasma with glass in the absence of 8HQ, followed by incubation with cellulose sulphate and quinoline, did not result in kinin activity while, reciprocally, pre-incubation of plasma with cellulose sulphate in the absence of 8HQ, rendered it unresponsive to subsequent activation with glass in the presence of 8HQ.

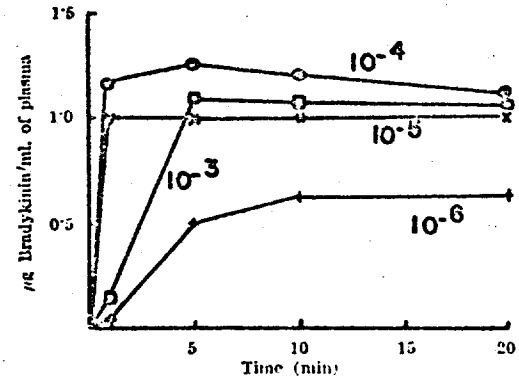

Fig. 1. Effect of concentration (w/v) of cellulose sulphate on the formation of bradykinin in oxalated rat plasma.

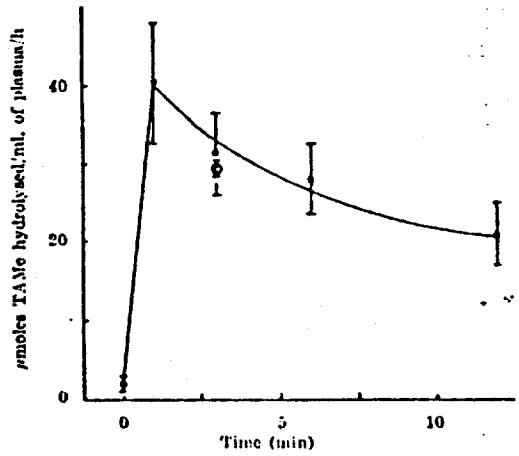

Fig. 2. Activation of TAMe esterase in human plasma by cellulose sulphate and glass powder. (Esterolytic activity was determined by the method of Brown[7].) ●, Cellulose sulphate, 1 mg/ml.; ○, glass powder, 500 mg/ml.

The bradykinin-forming activities of cellulose sulphate of agar and of heparin were also tested on oxalated human plasma. Their efficiency was approximately the same as those obtained in rat plasma, heparin being again the least active of the series.

Kinin formation by polysaccharide sulphates may involve activation of a proteolytic process. This was indicated by results showing that (*a*) soy-bean trypsin inhibitor (40 μg/ml. of plasma) prevented the action of every active polysaccharide tested, and (*b*) TAMe esterase activity in human plasma was markedly enhanced by cellulose sulphate (Fig. 2). Maximum activity, obtained after 1 min of incubation, was significantly decreased after 12 min. The nature of this inactivating effect is unknown but deserves further investigation. For comparison, Fig. 2 shows that an equivalent amount of esterolytic activity was present in plasma incubated for 3 min either with glass powder or with cellulose sulphate.

Polysaccharide sulphuric esters are prepared under comparatively mild conditions which do not cause changes in structure other than the introduction of sulphate substituents at hydroxyl groups[3]. This fact, as well as the high activity of this class of compounds, may make them useful tools in the study of the relationship between structure and activity as well as of chemical details of the kinin-forming process. These compounds, by decreasing bradykininogen stores *in vivo*, could, in addition, yield information about the participation of bradykinin in certain physiological or pathological processes[1].

We thank Professor M. Rocha e Silva for his helpful suggestions. This work was aided by grants from the U.S. Army Research Office and the National Research Council of Canada.

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Thromb. Diath. Haemorrh. 21(3), 1969.

The Influence of the Polysaccharide Carrageenin on the Clotting Mechanism of the Blood in the Rabbit in Vitro and in Vivo*

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Selye (1965) was able to sensibilize rats to cold with the high molecular polymer Carrageenin. After a preparatory injection he observed, upon exposure to cold, hemorrhagic necroses in the adrenals and in the acra (nose, paw, tail) and he also observed microcirculation disturbances in their immediate vicinity caused by cell-rich fibrinous thrombi. The histological changes were quite like those seen in the Sanarelli-Shwartzman-phenomenon (SSP) in the rabbit and were similar to those seen after the injection of the high molecular polymer polyanethol sulfate (Liquoid-Roche) in the rabbit (Rodriguez-Erdmann et al., 1960).

Characteristic of the clinical manifestations in the SSP and after Liquoid is a syndrome of thrombo-haemorrhagic tissue changes, circulatory shock and clotting disturbances. In both cases, an important member in the pathogenetic causal which leads to circulatory shock and to the tissue lesions is an intravascular activation of clotting followed by a consumption coagulopathy. One could also suspect participation of the clotting mechanism in animal experiments with sensibilisation by Carrageenin. Muth et al. (1967), stimulated by Selye, have investigated this question. After the injection of 55–100 mg of Carrageenin/kg body-weight in the rabbit they observed – even without exposure to cold – shock, consumption coagulopathy and intravascular clots in the lung, liver, heart, intestine, and kidneys. The analysis showed a decrease in clotting factors I (fibrinogen), II (prothrombin), V, VII, IX, X and a drop in thrombocytes. In the animals which had survived, the blood was found to be uncoagulable for several hours.

It is of interest that the consumption coagulopathy changes were observed in the experimental conditions described above, although a certain inhibitory effect on the clotting mechanism, similar to that of heparin, is attributed to Carrageenin as well as Liquoid. According to Hawkins and Leonhard (1962) the antithrombin activity of the natural Carrageenin mixture is, however, only about $^1/_{25}$ of the antithrombin activity of heparin. Either is this activity too small to prevent a consumption coagulopathy, or the inhibitory effect on clotting is of another nature than has hitherto been suspected. We have investigated this latter question in vitro and in vivo.

Method

Reagents

Carrageenin[1]) (Seakem 21 from Marine-Colloids Inc. Springfield, N. J., USA).

*) With the aid of a grant from the Deutsche Forschungsgemeinschaft.

1) Our thanks to Prof. H. Selye, who kindly provided us with this substance.

Carrageenin is a sulfatated polysaccharide, obtained mainly from the red alga chondrus crispus, which is used as a basis for cosmetics. It has an antilipemic effect and is also antipeptic and inhibits clotting. The molecular weight varies between 100,000 and 800,000. The commercially obtainable mixture contains Kappa-Carrageenin, which is a D-galactopyranosyl-3, 6-anhydro-D-galactopyranose-4-sulfate, and Lambda-Carrageenin, a galactopyranose-4-sulfate with a higher sulfur content than the Kappa-Carrageenin. The sulfate rests account for about 25% by weight and are bound to K, Na, and Ca as cations (quoted from Hawkins and Leonhard, 1962).

Parfentjew reagent. We mixed this as follows:
Ammonium sulfate 66.66
sodium chloride 5.0
merthylolate (ethyl-mercurithiosalicyllic acid sodium) 0.0125
aqua bi-destillata ad 500.0
The pH was adjusted to 7.0 with 10% NaOH.

FPT diluens for determination of fibrin polymerisation time containing kallikrein inhibitor according to Vermylen, De Vreker and Verstraete (1963, 1964). Obtained from: Manufacture de Produits Pharmaceutiques A. Christiaens s. a. Brussels.
Tris-HCl buffer (pH 7.4, ion strength 0.15)
Thrombin Behring-Werke Marburg/Lahn
factor V reagent Behring-Werke
Kinderfibrinogen Behring-Werke
Na citrate 3.8%
Na oxalate 3.4%
Potassium oxalate 25%
Kaolin Merck Darmstadt
Tachostyptan Hormon-Chemie Munich

Test Methods.

Thrombin time. The method with increasing thrombin concentrations was used according to Vermylen and Verstraete (1964).
To 0.1 ml of citrated plasma we added equal amounts of either
0.1 ml of physiological NaCl or
0.1 ml of heparin (0.5 u/ml) or
0.1 ml of Carrageenin (0.2 mg/ml)
In the series a), b) and c) the thrombin time is determined with a series of different concentrations ranging from 0.375 u of thrombin/ml of plasma, 0.75 u, 1.5 u, 3 u, to 6 u of thrombin/ml of plasma.

Protamine titration. The thrombin time of 0.1 ml of citrated plasma which contains either 0.1 ml of sodium chloride or 0.1 ml of Carrageenin (final concentration 0.2 mg/ml plasma) is determined in the presence of different protamine sulfate concentrations.

Fibrin polymerisation time. According to Vermylen, De Vreker and Verstraete (1963, 1964).

Determination of factor V. One-phase method with the reagent from the Behring-Werke Marburg/Lahn.

Determination of factor VIII. One-phase method according to Jung and Duckert (1960).

Determination of antithrombin III. According to Gerendas (1949, 1960). The oxalated plasma containing antithrombin was always defibrinated at 56° C for 3 min.

Anti-plasma thrombokinase. Determination according to Egli, Kesseler and Klesper (1957), with the modification according to Schimpf, Gramlich and Türk (1960).

Fibrinogen precipitation with Parfentjew reagent.
0.5 ml of citrated plasma to which are added
4.5 ml of the Parfentjew reagent. Parallel to this as a control
0.5 ml of citrated plasma to which is added
4.5 ml of physiological sodium chloride.

The glasses are rocked slowly to obtain thourough mixing. After precisely 3 min the opacity in each glass is measured at 510 millimicrons and the fibrinogen concentration obtained from a standard reference curve.

Procedure in vivo and in vitro:

In vivo we gave the rabbits a dose of 10 mg of Carrageenin/kg body-weight, since the dose of 55 mg/kg bodyweight used by Huth et al. (1967) was too high for our animals. With the dose of Huth et al. the animals died within a few minutes. This could be the result of an increased sensitivity due to a different season of the year or simply because our rabbits were of another race. Into the ear vein of a rabbit weighing 3 kg we injected 30 mg of Carrageenin in 15 ml of sodium chloride within the space of 10 min.

For determination blood was withdrawn through an ear vein catheter according to Rodriguez-Erdmann (1959) shortly before and 15 min after the injection of 10 mg of Carrageenin/kg body-weight; in further series samples were withdrawn immediately before and 2 hrs after injection.

In vitro we always added 0.2 mg of Carrageenin/ml of plasma according to the following approximation: assuming a blood volume of approximately 100 ml/kg rabbit, this means that 10 mg of Carrageenin/kg body-weight equal 10 mg of Carrageenin in 100 ml of blood. That is 0.1 mg in 1 ml of blood or in approximately 0.5 ml of plasma. That equals 0.2 mg of Carrageenin in 1 ml of plasma.

In each determination equal amounts of a solution containing 0.2 mg of Carrageenin (per ml of sodium chloride) and of plasma, in the case of the controls equal volumes of sodium chloride and plasma, were incubated for 15 min or 2 hrs at 37° C.

Results

A. Experiments in Vitro

1. Influence of Carrageenin on the determination of thrombin time with increasing thrombin concentrations.

Three series of experiments were carried out:

a) with the addition of a constant concentration of Carrageenin,
b) with the addition of physiological sodium chloride,
c) with the addition of a constant concentration of heparin.

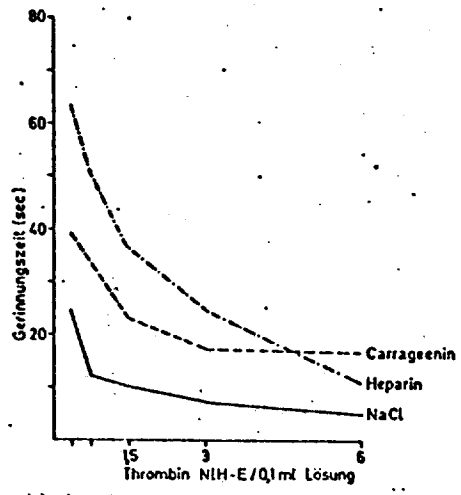

Fig. 1. Thrombin time with changing thrombin concentration in the presence of constant concentrations of sodium chloride, Carrageenin or heparin in vitro. Ordinate: clotting time in sec. Abscissa: thrombin-N.I.H.U./0.1 ml.

The thrombin time observed with the addition of heparin approaches the sodium chloride control value with an increasing concentration of thrombin (see Fig. 1). That is to say, with a constant heparin concentration increasing amounts of thrombin are able to overcome more and more the inhibitory effect of heparin on the thrombin activity and finally to eliminate it completely. Heparin is an antithrombin.

With the addition of Carrageenin the thrombin time never approaches the thrombin time observed with the addition of sodium chloride in spite of increasing thrombin concentration. In the presence of Carrageenin the thrombin time constantly remains about 12 sec longer than in the presence of sodium chloride even with high thrombin concentrations. Thus, Carrageenin inhibits a process which takes place only after thrombin has taken effect: that is to say: fibrin aggregation or polymerisation. High doses of thrombin are not able to counteract this effect. The shape of the curve in Fig. 1 can be taken as proof that heparin is an antithrombin, whereas Carrageenin is an antipolymerase.

2. The influence of protamine on the thrombin time determination in the presence of Carrageenin.

It is shown that the inhibitory effect of Carrageenin on clotting is almost completely eliminated by protamine. Strangely enough, the protamine concentration in the amounts we used is of no importance. The shortest clotting time in the thrombin time determination is found with equal protamine concentrations (see Figs. 2, 3), irrespective of whether Carrageenin or physiological sodium chloride had been added to the plasma. This weighs against heparin-like properties of Carrageenin.

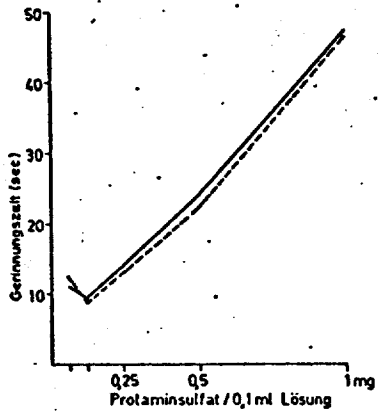

Fig. 2.

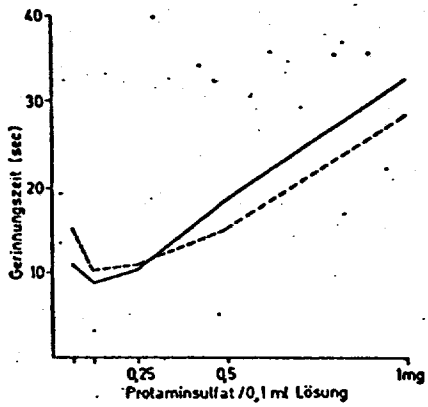

Fig. 3.

Fig 2. Protamine titration in vitro. ——— 15 min after incubation with NaCl, - - - - - 15 min after incubation with Carrageenin. Ordinate: Clotting time in sec, Abscissa: mg of protamine sulfate/0.1 ml.

Fig 3. Protamine titration in vitro after Carrageenin in vivo. ——— before injection, - - - - - 15 min after injection of Carrageenin. Ordinate: Clotting time in sec, Abscissa: mg protamine sulfate/0.1 ml.

3. Fibrinpolymerisation time according to Verstraete and Vermylen (1963/64).

This one-phase test, too, shows an increase in the clotting time of fibrinogen in the presence of Carrageenin-containing plasma (which has been previously heated to 56° C

for 3 min). The thrombin concentration in this test system is so high that antithrombins in the added test plasma are not able to exert any effect upon the clotting time of the substrate fibrinogen (see Fig. 4).

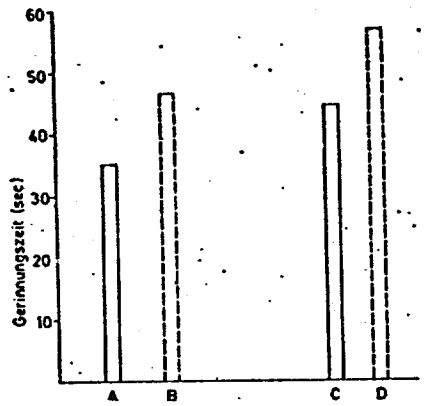

Fig. 4.

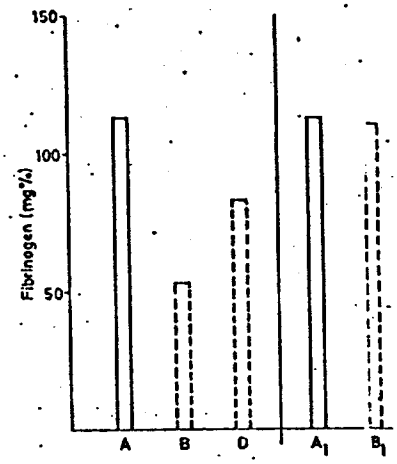

Fig. 5.

Fig. 4. Fibrinpolymerisation time in vitro. A = 15 min after incubation with NaCl, B = 15 min after incubation with Carrageenin, C = 2 hrs after incubation with NaCl, D = 2 hrs after incubation with Carrageenin. Ordinate: Clotting time in sec.

Fig. 5. Fibrinogen. *In vivo:* A = before injection, B = 15 min after injection of Carrageenin, D = 2 hrs after injection of Carrageenin. *In vitro:* A_1 = 15 min after incubation with NaCl, B_1 = 15 min after incubation with Carrageenin. Ordinate: Content of fibrinogen in mg%.

B. Comparisons in Vivo and in Vitro

In principle, an antipolymerase increases not only the clotting time in thrombin time determination but in all clotting tests in which the fibrinpolymerisation phase is involved. Depending on the test, this increase simulates a higher or lower activity of the investigated clotting factor. For this reason, we felt it pertinent to investigate the influence on the determinations of factors I, V, VIII; antithrombin III and antiblood-thromboplastin in vitro, in order to evaluate the changes caused by Carrageenin in vivo.

1. Fibrinogen

In vitro: Incubation of plasma with 0.2 mg of Carrageenin/ml effects only a minimal fall in the fibrinogen level (see Fig. 5). The determination of fibrinogen according to Parfentjew is not affected by antipolymerases.

In vivo: It can be seen that this fall in fibrinogen after Carrageenin i. v. is real (Huth et al., 1967). In our tests, 15 min after injection fibrinogen had fallen from 113 mg/100 ml to 53 mg/ml 100 and after 2 hrs had risen again to 84 mg/100 ml (see Fig. 5).

2. Factor VIII

In vitro: 15 min after the incubation with sodium chloride at 37° C the activity of factor VIII was 115%, and 2 hrs after incubation with sodium chloride 25%. 15 min

after the incubation with 0.2 mg of Carrageenin/ml the factor VIII activity was only 21%, and after 2 hrs of incubation only 7.8% (see Fig. 6).

It is probable that the antipolymerase effect of Carrageenin simulates a direct effect on factor VIII.

In vivo: 15 min after the injection of 10 mg of Carrageenin/kg body-weight i. v. the activity of factor VIII had fallen from 115 to 2.35%, and 2 hrs after injection had climbed again to 47% (see Fig. 6).

The greater drop in factor VIII activity after 15 min in vivo as compared with in vitro possibly can be ascribed to the consumption reaction due to Carrageenin.

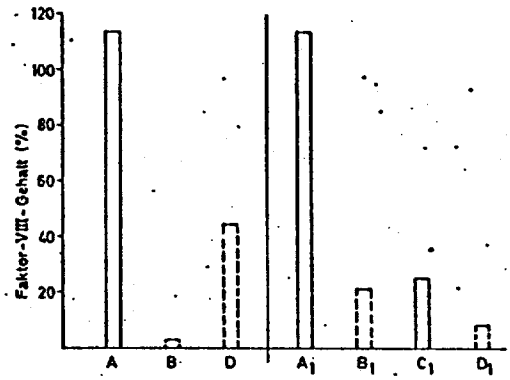

Fig. 6. Factor VIII activity. *In vivo:* A = before injection. B = 15 min after injection of Carrageenin, D = 2 hrs after injection of Carrageenin. *In vitro:* A_1 = 15 min after incubation with NaCl, B_1 = 15 min after incubation with Carrageenin, C_1 = 2 hrs after incubation with NaCl, D_1 = 2 hrs after incubation with Carrageenin. Ordinate: Factor VIII activity.

3. *Factor V*

In vitro: 15 min after incubation with sodium chloride the activity of factor V had fallen to 97%, 2 hrs after incubation with sodium chloride to 40%. 15 min after incubation with Carrageenin the activity of factor V had fallen to 44%, and after 2 hrs of incubation with Carrageenin to 5,5% (see Fig. 7).

Here, too, one could assume that the antipolymerase effect of Carrageenin simulates a drop in factor V activity.

In vivo: 15 min after 10 mg of Carrageenin/kg body-weight i. v. the level of factor V activity was 100% instead of 115%, 2 hrs after injection it was still 90% (see Fig. 7).

This minimal drop does not speak against the possibility that a consumption reaction had taken place in vivo. It is rather dependent upon the extent of the reaction, if factors such as fibrinogen, factor V and factor VIII all show the same obvious degree of drop or if one or the other factor only varies within its normal range.

On the other hand, these results concerning factor V show that the seeming drop in factors in vitro is not due to a direct influence upon the factor V molecule by Carrageenin. In such a case one could expect the effect to be about the same after the injection in vivo.

4. *Antithrombin III*

If thrombin and plasma containing Carrageenin were incubated together according to Gerendas (1949, 1960), and if samples from the incubation mixture were added to

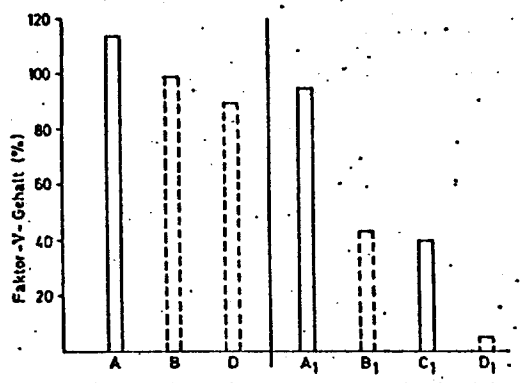

Fig. 7. Factor V activity. *In vivo:* A = before injection, B = 15 min after injection of Carrageenin, D = 2 hrs after injection of Carrageenin. *In vitro:* A_1 = 15 min after incubation with NaCl, B_1 = 15 min after incubation with Carrageenin, C_1 = 2 hrs after incubation with NaCl, D_1 = 2 hrs after incubation with Carrageenin. Ordinate: Factor V activity in %.

substrate fibrinogen, the clotting times were clearly longer than in the absence of Carrageenin. This lengthening, however, remained always of the same degree and was independent of the length of the incubation time of thrombin and Carrageenin-containing plasma before the addition to the substrate fibrinogen. The inactivation of thrombin by antithrombin III was thus neither accelerated nor retarded by Carrageenin (see Fig. 8). There were no differences in vivo and in vitro.

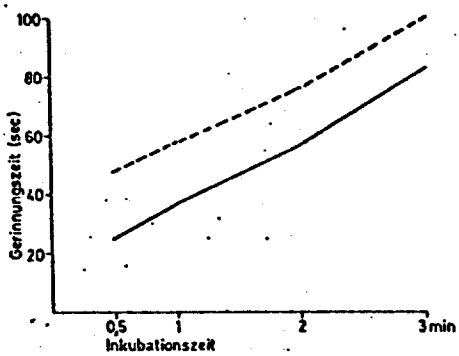

Fig. 8.

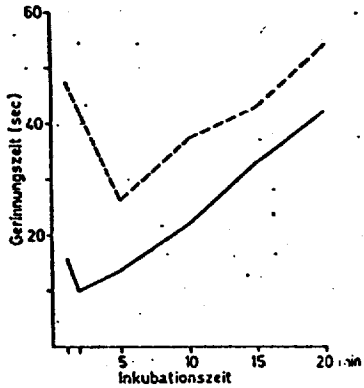

Fig. 9.

Fig. 8. Antithrombin III in vivo. ——— before injection, - - - - - - 15 min after injection of Carrageenin. Ordinate: Clotting time in sec, Abscissa: Incubation time in min.

Fig. 9. Anti-bloodthromboplastin in vitro. ——— 15 min after incubation with NaCl, - - - - - - 15 min with Carrageenin. Ordinate: Clotting time in sec, Abscissa: Incubation time in min.

5. *Anti-plasmathromboplastin*

Here the result was in principle the same as in the determination of antithrombin III. The inactivation of plasmathromboplastin by plasma containing anti-plasmathromboplastin

plastin was neither accelerated nor retarded. Only the clotting time of the substrate plasma was consistently lengthened by the same amount (see Fig. 9).

There was no difference in vivo and in vitro here, either.

5. *Fibrinpolymerisation time (see also section A_3)*

In vivo and in vitro a lengthening was observed due to Carrageenin. In vivo 2 hrs after injection the effect is smaller than after 2 hrs of incubation in vitro. One must assume that the Carrageenin level in vivo had fallen after 2 hrs (see Fig. 10, 4).

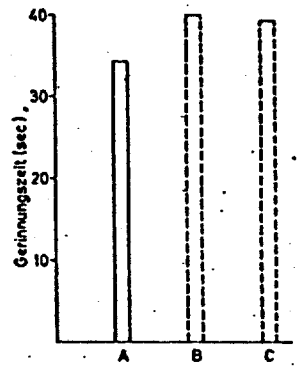

Fig 10. Fibrinpolymerisation time in vivo. A = before injection, B = 15 min after injection of Carrageenin, C = 2 hrs after injection of Carrageenin. Ordinate: Clotting time in sec.

Discussion

Carrageenin has no antithrombin effect. It inhibits fibrin aggregation or polymerisation. Its coagulation-inhibitory component can be blocked by protamine. However, this effect is independent of the protamine concentration. This shows that no heparin-like property exists. That had been hitherto assumed, since Carrageenin as well as heparin lengthens the thrombin time (Hawkins and Leonhard, 1962, 1963).

Despite this inhibitory effect on clotting a thrombohaemorrhagic syndrome can be induced by the injection of Carrageenin (Selye, 1965), and a consumption coagulopathy can be produced (Huth et al., 1967). One wonders: Why does Carrageenin not counteract its own ability to induce a consumption reaction by means of its clotting-inhibitory component?

To make blood absolutely uncoagulabel it takes 2 u of heparin/ml (Lyttleton, 1954) or approx. 0.02 mg of heparin/ml. Since according to Hawkins and Leonhard (1962, 1963) heparin is 25 times stronger than Carrageenin, to produce uncoagulability with Carrageenin it should take 0.02 mg time 25 which is equal to 0.5 mg/ml or 50 mg of Carrageenin/100 ml of blood (in our own experience, however, 10 mg/100 ml of blood or less is sufficient). The dose of 50 mg/100 ml in our rabbits caused immediate death. The animals of Huth et al. (1967), however, survived levels of 55 mg of Carrageenin/100 ml and more. In spite of this a consumption coagulopathy was not prevented by the clotting-inhibitory properties of this high Carrageenin concentration.

This contradiction is, in our opinion, due to the special properties of Carrageenin: since it is not an antithrombin, it is not able to immediately reverse a consumption reaction which it itself has initiated. The formation of free thrombin is not prevented

and, for example, the viscous metamorphosis of thrombocytes can be initiated. The coagulability of the remaining circulating blood is now doubly inhibited:
1. by the consequence of the consumption reaction and
2. by the inhibitory properties of Carrageenin.
Probably the same considerations can be applied to the effect of Liquoid (Hausmann and Dreyfus, 1953; Rodriguez-Erdmann et al., 1960).

Summary

Carrageenin is not an antithrombin. It inhibits fibrin aggregation or polymerisation. The inactivation of thrombin by antithrombin and of blood thromboplastin by anti blood thromboplastin is neither accelerated nor retarded. In the presence of Carrageenin the clotting times of tests in which the fibrinpolymerisation phase is involved are lengthened; this simulates a decrease in clotting factors. In vivo Carrageenin, despite its coagulation-inhibitory properties, is not able to reverse the consumption reaction which it has initiated.

Résumé

Le Carrageenin n'est pas une antithrombine. Il inhibe l'agrégation et la polymérisation de la fibrine. Ni l'activation de la thrombine par l'antithrombine ni celle de la thromboplastine sanguine par l'antithromboplastine ne sont accélérées ou retardées. Le Carrageenin in vivo, malgré ses propriétés inhibitrices n'est pas capable de bloquer les réactions de consommation qu'il a mises en marche. En présence de Carrageenin les temps de coagulation sont prolongés chaque fois que la polymérisation de la fibrine joue un rôle. Cela simule une diminution des facteurs de coagulation.

Zusammenfassung

Carrageenin ist kein Antithrombin. Es hemmt die Fibrinaggregation oder -polymerisation. Die Inaktivierung von Thrombin durch Antithrombin und von Blutthromboplastin durch Anti-Blutthromboplastin wird weder beschleunigt noch verzögert. In der Gegenwart von Carrageenin werden die Gerinnungszeiten von Tests, bei denen die Phase der Fibrinpolymerisation durchlaufen wird, verlängert; dies täuscht einen Abfall von Gerinnungsfaktoren vor. In vivo kann Carrageenin die von ihm angestoßene Verbrauchsreaktion trotz seiner gerinnungshemmenden Eigenschaften nicht unterdrücken.

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Carrageenan and Delayed Hypersensitivity
II. Activation of Hageman Factor by Carrageenan and Its Possible Significance[1] (34358)

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Carrageenan is a high molecular weight, long-chain polymer of sulfated galactose units that can be extracted from marine plants. Carrageenan has recently been shown to suppress the delayed hypersensitivity reactions of sensitized guinea pigs (1), inhibit the complement system (2, 3), damage macrophages *in vitro* (4) and *in vivo* (5), and induce kinin formation in rat plasma (6).

In this paper we show that carrageenan activates Hageman factor (factor XII) in human plasma, and thus promotes blood coagulation, induces vascular permeability, and liberates kinin (7). At higher concentrations it also acts as an anticoagulant, as reported previously (8).

Materials and Methods. Carrageenan was kindly supplied by Marine Colloids, Inc., Springfield, New Jersey as Seakem 21. The powder was dissolved in hot saline prior to use, using a double boiler technique.

Human platelet-deficient plasma was prepared in silicone coated plastic equipment. Venous blood was drawn into 30-ml plastic syringes, mixed with 0.1 vol of 0.13 *M* sodium citrate pH 5.0 in 40-ml Lusteroid tubes and centrifuged 30 min at 700*g* at 1°. Plasma was then transferred to thick-walled silicone coated Servall centrifuge tubes and centrifuged at 30,000*g* for 30 min. Plasma aliquots were then transferred to plastic, silicone coated containers and stored in the frozen state until use.

PTA-deficient, Christmas factor-deficient, and Hageman factor-deficient plasma was kindly provided by Dr. Oscar D. Ratnoff.

Barbital–saline buffer and DeJalon solution was prepared as described elsewhere (9, 10). *Soybean trypsin inhibitor* (SBTI) (Worthington) was freshly prepared in barbital–saline buffer at desired concentrations. *O*-Phenanthroline (Fisher) was prepared in barbital–saline buffer (2.5×10^{-3} *M*) and stored at 4° for less than 2 weeks. *Hexadimethrene bromide* (Polybrene, Abbott Laboratories) was similarly prepared and stored. *Anti-Hageman factor globulin* was prepared as described previously (11) and kindly provided by Dr. Oscar D. Ratnoff.

Vascular permeability enhancing activity was assessed by calculating the mean diameter (mm) of the blue spot developed after intradermal injection of 0.1 ml of test material into each of 4 guinea pigs pretreated with Pontamine sky blue (11, 12).

Recalcified clotting time of platelet-deficient plasma was measured in 10 × 75-mm polystyrene test tubes (Falcon) in duplicate as previously described (13).

Development of kinin activity in plasma was measured by methods previously described (14), using the isolated rat uterus.

Results. I. The effect of carrageenan on blood clotting. Carrageenan had a dual, dose-dependent effect on the recalcified clotting time of normal human plasma. Anticoagulant activity was seen at concentrations greater than 10^{-3} mg/ml, whereas procoagulant activity was seen at lower concentrations. Maximum procoagulation was seen at 3.3×10^{-5} mg/ml, at which level the recalcified clotting time was reduced to approximately ½ of the control (Table I).

When plasma deficient in Hageman factor was used, prolonged clotting times were again noted at high concentrations (Table II) and

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TABLE I. The Effect of Carrageenan on the Recalcified Clotting Time of Normal Human Platelet-Deficient Plasma.

Test substance		Clotting time (min)
Buffer		28.5
Carrageenan* (mg/ml)	3.3	>75.0
	3.3×10^{-1}	>75.0
	3.3×10^{-2}	>75.0
	3.3×10^{-3}	34.0
	3.3×10^{-4}	22.7
	3.3×10^{-5}	12.0
	3.3×10^{-6}	17.0
	3.3×10^{-7}	20.5
	3.3×10^{-8}	22.7

* Final concentrations in a total volume of 0.3 ml containing 0.1 ml of plasma and 0.1 ml of $CaCl_2$.

only slight decrease in the recalcified clotting time was seen at lower concentrations. No clot-promoting activity was seen in cow plasma deficient in PTA, or in human plasma deficient in Christmas factor.

When hexadimethrene bromide (2×10^{-1} and 5×10^{-2} mg/ml) was added to normal human plasma, the procoagulant effect of carrageenan was blocked. This effect was less apparent at a concentration of 2×10^{-2} mg/ml (Table III).

II. The effect of carrageenan on permeability enhancing activity in plasma. Incubation of carrageenan (3.3×10^{-4} mg/ml) with normal human plasma led to the development of permeability enhancing activity (Table IV). This effect was prevented by the prior addition of SBTI (Table IV) to normal plasma, and was not obtained when Hageman factor-deficient plasma was used. The intradermal injection of carrageenan (3.3×10^{-1}) alone did not lead to increased vascular permeability.

III. The effect of carrageenan on kinin formation. Incubation of carrageenan (3.3×10^{-1}) with normal human plasma led to activation of the kinin system, as measured by smooth muscle contraction in the rat uterus assay system (Table V), This result was abolished by addition of SBTI to the incubation mixture.

When carrageenan was incubated with Hageman factor-deficient plasma, kinin activity did not develop. When normal human plasma was preincubated with anti-Hageman factor globulin, and carrageenan (3.3×10^{-1} mg/ml) later added, the development of kinin activity was suppressed, with only ⅓ of the control contraction attained.

Discussion. It has been shown that activation of Hageman factor in normal human plasma results in accelerated blood clotting, and in the development of both kinin and vascular permeability enhancing activity as well (7). In the present paper we confirmed previous reports that carrageenan has anticoagulant (8) and kinin-releasing activities (6). In addition, we showed that carrageenan can also act as a procoagulant and can bring about enhanced vascular permeability.

We feel that these latter three effects are the result of the activation of Hageman factor by carrageenan. They develop in normal plasma, but fail to develop in Hageman factor-deficient plasma. SBTI and hexadimethrene bromide are known to interfere with the consequences of Hageman factor activation (Ref. 7). Thus, the suppression of the procoagulant, kinin releasing, and vascular permeability enhancing activities in normal plasma of carrageenan by these materials (Tables III–V) and the inhibition of kinin activation by anti-Hageman factor globulin (Table V) are consistent with this interpretation.

Carrageenan has several other interesting biological effects as well. It inhibits the complement system, by interfering with the capacity of C′1 to become bound to sensitized

TABLE II. The Effect of Carrageenan on the Recalcified Clotting Time of Hageman Factor-Deficient Human Platelet-Deficient Plasma.

Test substance		Clotting time (min)
Buffer		69.0, 72.0
Carrageenan* (mg/ml)	3.3×10^{-1}	>120.0, 115.0
	3.3×10^{-3}	40.5, 51.5
	3.3×10^{-5}	31.0, 42.0
	3.3×10^{-6}	38.0, 43.0
	3.3×10^{-7}	54.0, 49.5
	3.3×10^{-8}	48.5, 46.5

* Final concentrations.

TABLE III. The Effect of Hexadimethrene Bromide on the Clot-Promoting Activity of Carrageenan.

Mixture	Recalcified clotting time of normal plasma[a] (min)
Buffer + normal plasma	24, 26
Carrageenan (2.5×10^{-4} mg/ml) + normal plasma	11, 11
Hexadimethrene (5×10^{-2} mg/ml) + normal plasma	85, 85
+ carrageenan (2.5×10^{-4} mg/ml) + normal plasma	85, 85
(1.25×10^{-2} mg/ml) + normal plasma	50, 52
+ carrageenan (2.5×10^{-4} mg/ml) + normal plasma	50, 52
(5×10^{-3} mg/ml) + normal plasma	31, 31
+ carrageenan (2.5×10^{-4} mg/ml) + normal plasma	23, 25

[a] In a total volume of 0.4 ml, containing 0.1 ml of each reagent plus sufficient buffer to make up to final volume. Concentrations indicated are final in volume used.

erythrocytes (3). It also is an immunogen, causing the formation of precipitating antibodies in rabbits (15), and in a growth stimulant for connective tissue (16).

Of what biological significance may these properties of carrageenan be? Davies showed no alteration in the susceptibility of carrageenan-treated animals to anaphylaxis, although a significant decrease in serum complement levels were observed (2). It has been demonstrated previously that administration of carrageenan to tuberculin-sensitive guinea pigs results in a dose-dependent suppression of delayed hypersensitivity which was not correlated with anti-complementary or anticoagulant activity (1). It was suggested that the suppressed delay hypersensitivity reactions resulted from the toxic effect of carrageenan on macrophages (1).

Current immunologic theory explains the evolution of a delayed hypersensitivity reaction as a two-step process (17). The first event is the interaction between antigen and specifically sensitized small lymphocytes. This interaction may lead to release of chemotactic and other humoral factors which bring about local vascular changes, and an accumulation of macrophages. After a period of hours, the typical delayed, or tuberculin-type reaction, is observed (18).

The inhibition of delayed hypersensitivity reactions by carrageenan may be due to a direct toxic action of this compound on macrophages (1, 5). It may be, however, that its ability to activate the clotting, kinin, interrelated systems via its effect on Hageman factor may be responsible, at least in part, for this inhibitory action. Kellermeyer and

TABLE IV. The Effect of Carrageenan on Permeability Enhancing Activity of Human Plasma.

Mixture tested[a]	Permeability activity (mm)[b]
Normal plasma + buffer	2.4
+ carrageenan (3.3×10^{-4} mg/ml)	7.0
Hageman factor-deficient plasma + buffer	1.9
+ carrageenan (3.3×10^{-4} mg/ml)	2.0
Normal plasma + soybean trypsin inhibitor (0.25 mg/ml)	1.9
+ carrageenan (3.3×10^{-4} mg/ml) + soybean trypsin inhibitor[c] (0.25 mg/ml)	1.2
Carrageenan (3.3×10^{-4} mg/ml) + buffer	1.3

[a] Concentrations are for final volume.

[b] These numbers represent the average diameter of the blue spot developed after intradermal inoculation of 0.1 ml of test material into each of four guinea pigs preinjected with Pontamine sky blue.

[c] Soybean trypsin inhibitor added prior to carrageenan.

TABLE V. The Effect of Carrageenan on Kinin Activity Formation in Human Plasma.

Mixture tested	Kinin releasing activity[a]
Normal plasma + buffer + phenanthroline	0
+ carrageenan (3.3×10^{-1} mg/ml) + phenanthroline	+
Hageman factor-deficient plasma + carrageenan (3.3×10^{-1} mg/ml) + phenanthroline	0
Normal plasma + soybean trypsin inhibitor + phenanthroline + carrageenan (3.3×10^{-1} mg/ml)	0
Normal plasma + anti-Hageman factor globulin[b] + phenanthroline + carrageenan (3.3×10^{-1} mg/ml)	⅓
Normal plasma + buffer + phenanthroline + carrageenan (3.3×10^{-1} mg/ml)	+

[a] + = maximal contraction; 0 = no contraction; ⅓ represents a contraction 67% less than maximal.
[b] Normal plasma incubated with antiserum at 37° for 3 hr; carrageenan added for 15 min, then mixture was tested.

Warren recently found that the granuloma response to schistosome ova is inhibited when mice are pretreated with ellagic acid (an activator of Hageman factor) (13, 19). This granuloma model has many characteristics of a delayed hypersensitivity reaction (20). The suppression of two varieties of delayed hypersensitivity by materials which activate Hageman factor suggests the possibility that one of the Hageman factor-dependent sequences may play a role in delayed hypersensitivity reactions. Intravenous injection of ellagic acid resulted in kinin formation and kininogen depletion in rats (21). It thus seems possible that the effects of carrageenan and ellagic acid in delayed hypersensitivity reactions may in part be explained by a consumptive depletion of these factors. Further studies along these lines are now in progress.

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THE EFFECT OF CARRAGEENAN ON DELAYED HYPERSENSITIVITY REACTIONS[1]

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Administration of carrageenan to tuberculin-sensitive guinea pigs resulted in a dose-dependent suppression of delayed hypersensitivity. Recovery was complete 1 week after drug administration. The suppressive activity was not correlated with anticomplementary or anticoagulant activity. It is suggested that carrageenan exerts its effect as a result of its toxicity for macrophages, acting through lysosomal damage and the release of intracellular enzymes.

Several lines of evidence suggest that the expression of delayed hypersensitivity is a two step process (1). The first event is the interaction between specifically sensitized small lymphocytes and specific antigen at the local skin test site. Passive transfer studies have demonstrated that only a relatively few specifically sensitized lymphocytes appear at this site (2). Following such local interaction, and perhaps as a result of the release of humoral factors, nonspecific mononuclear cells enter the reaction area and after a period of hours make up the typical delayed skin reaction (3). Turk *et al.* (4, 5) subdivided mononuclear cells in dermal delayed hypersensitivity reactions in two groups, macrophages and lymphocytes, depending on their content of lysosomes, as demonstrated by histochemical staining for acid phosphatase. Delayed hypersensitivity reactions to tuberculin, diphtheria toxoid and human γ globulin have their peak intensity at 24 hr in the guinea pig and at that time were shown to consist of approximately 50% macrophages.

Carrageenan is a long chain, high molecular weight sulfated polygalactose derived from marine plants. Allison *et al.* (6) have reported that it is toxic to macrophages in an *in vitro* system, apparently by being taken up rapidly and causing the release of secondary lysosomal enzymes. Similar effects have also been attributed to silica (6).

This study represents an effort to use these effects to assess the contribution of macrophages in the *in vivo* skin reaction.

MATERIALS AND METHODS

Random-bred male albino guinea pigs weighing 300 g were immunized with 0.1 ml of Freund's complete adjuvant (containing light mineral oil, Arlacel A and *Mycobacterium tuberculosis*, 5 mg/ml) distributed among the four footpads. Three to 4 weeks later the animals were shaved, depilated and skin tested with 1:200 old tuberculin (O.T.) in 0.1 ml of saline. Test sites were observed at 3 hr for evidence of Arthus reactions and measured at 24 hr for induration and erythema. Reactions were graded according to diameter of erythema in millimeters and intensity of induration: − = no induration, + = slight induration, ++ = induration, +++ = central blanch.

Calcium carrageenan (Seakem 21, Marine Colloids, Inc., Springfield, N. J.) was injected as a saline solution made in concentrations needed at the time of use.

Viscarin (Marine Colloids, Inc.) is partially purified λ carrageenan and was freshly prepared in saline for use.

Gelcarin (Marine Colloids, Inc.) is partially purified κ carrageenan used in freshly prepared saline solutions for these studies.

Hirudin (Sigma Chemical Corporation, St. Louis, Mo.), an extract of leeches with anticoagulant properties, was used in freshly prepared saline solutions containing 270 units/mg.

Heparin (Liquamine Sodium, Organon, Inc.,

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West Orange, N. J.) was used in solutions containing 1000 units (10 mg)/ml.

MIN-U-SIL (Pennsylvania Glass Sand Corporation, Pittsburgh, Pa.), which consists of 5-μ particles of silica, was administered as a 20-mg/ml suspension.

All materials used in these studies were administered i.p. Controls were given saline to assess the nonspecific effects of i.p. injections.

Effect of drugs on coagulation. In some experiments, whole blood clotting time determinations were carried out using 2 ml of whole blood obtained by cardiac puncture in each of three test tubes. These determinations were made on separate animals, at either 2, 4 or 24 hr after initiation of drug treatment.

Determination of vascular permeability. The effect of carrageenan on the vascular component of delayed hypersensitivity was studied by observing the area of leakage of Evans blue dye given i.v. 12 hr after skin testing, in a dose of 3 mg/100 g of animal as a 2.5% solution in saline. Readings were made 30 min after administration and the diameter of the resulting blue area was measured in millimeters.

Estimation of serum complement levels. CH_{50} levels were determined in the laboratories of Dr. K. Frank Austen.

RESULTS

In Table I are seen the results of administering various doses of carrageenan to tuberculin-sensitive guinea pigs at the time of skin testing with 1:200 O.T. At doses of 10 mg some inhibition of induration became apparent and there was progressive increase in this inhibitory effect with increasing doses up to 100 mg, where virtually all induration and erythema disappeared. In this experiment, tuberculin sensitivity was particularly intense and control reactions were over 25 mm with intense induration and central blanching. In subsequent experiments lower levels of sensitivity were studied by using guinea pigs within 3 weeks of sensitization. With milder reactions more striking suppression could be obtained with 10-mg doses of carrageenan.

In the next experiment (Table II) the effect of time of administration of carrageenan was evaluated. A 10-mg dose at the time of skin testing produced total suppression of induration and diminution in erythema as well. This dose produced a similar effect when given 4 hr before skin testing, but when given 16 hr before considerably less effect was noted. However, a larger dose (50 mg) produced marked suppression when given 16 hr prior to skin testing. It is of some interest that neither dose produced any change when administered 5 hr after skin testing.

The suppressive effects of carrageenan were transient and repeated skin tests demonstrated partial return of reactivity 3 days later and full return at the end of a week.

As far as systemic effects were concerned, carrageenan produced no marked changes in the total white blood cell and differential counts in

TABLE I

Relation between dose of carrageenan administered at time of skin test and tuberculin reaction at 24 hr

Dose of Carrageenan	Delayed Reactions[a] to O.T. 1:200
mg	
1.0	25 +++, 21 +++, 18 +++, 15 +++, 16 +++, 20 +++
5.0	24 +++, 26 +++, 19 +++, 18 +++, 17 +++, 17 +++
10.0	22 +++, 20 +++, 27 +++, 18 ++, 16 ++, 12 ++
50.0	12 ++, 15 ++, 11 ++, 18 ++, 10 ++, 6 −
100.0	5 −, 8 −, 6 −, 0, 0, 0, 0
Control	20 +++, 25 +++, 24 +++, 26 +++

[a] Millimeters of erythema and degree of induration: − = no induration, + = slight induration, ++ = induration, +++ = central blanch.

TABLE II

Relationship between time of carrageenan administration and tuberculin reaction

Treatment with Carrageenan	No. of Animals	Average Delayed Reaction[a] to O.T. 1:200
10 mg at time 0	8	6 −
10 mg at −4 hr	5	6 −
10 mg at −16 hr	6	12 ++
10 mg at +5 hr	6	16 ++
50 mg at −16 hr	7	7 −
50 mg at +5 hr	8	16 ++
Saline at 0 hr	20	17 ++

[a] Millimeters of erythema and degree of induration: − = no induration, + = slight induration, ++ = induration.

TABLE III

Relationship between suppression of tuberculin reactivity and anticoagulant effect

Treatment	No. of Animals	Average Delayed Reaction[a] to O.T. 1:200	Clotting Time
			min
Gelcarin, 10 mg × 1	5	4 ±	4
Viscarin, 10 mg × 1	5	5 ±	5
Carrageenan, 10 mg × 1	3	5 ±	4
Hirudin, 2 mg q6h × 4	5	11 +	8
Heparin, 10 mg q6h × 4	5	15[b] +	45
Saline	12	15 ++	4

[a] Millimeters of erythema and degree of induration; − = no induration, + = slight induration, ++ = induration.

[b] Erythema markedly decreased.

TABLE IV

Effect of carrageenan on vascular permeability in tuberculin reactions

Treatment	Delayed Reaction[a] to O.T. 1:200 at 12 Hr	Diameter of Blueing 30 Min after Dye Injection
Carrageenan, 10 mg i.p.	14 ±	15
	7 −	9
	12 ±	15
	15 −	8
	11 −	12
Saline	19 ++	13
	15 ++	11
	15 ++	17

[a] Millimeters of erythema and degree of induration: − = no induration, + = slight induration, ++ = induration.

the peripheral blood, nor were any significant decreases in serum complement (CH_{50}) levels noted in animals exhibiting marked suppression of delayed reactions. Because of the observed effect of anticoagulants on delayed hypersensitivity (7) and because carrageenan is a sulfated polygalactose, the clotting times of whole blood from treated and control animals were compared. By simple determinations in tubes, Lee-White clotting times of 3 to 6 min were found for both groups.

In a further series of experiments to test the possible relationship between anticoagulant action and inhibitory efficacy, different groups of guinea pigs were treated with the anticoagulants heparin and leech hirudin as well as the variously sulfated polysaccharides carrageenan, gelcarin and viscarin. As can be seen in Table III, a single injection of 10 mg of viscarin, gelcarin or carrageenan gave marked suppression of delayed reactivity in the face of normal clotting times. Hirudin administered in 2-mg doses every 6 hr only slightly suppressed reactivity with some increase in clotting time. Heparin administered in 10-mg doses every 6 hr starting 0.5 hr before skin tests caused a marked decrease in erythema, but little reduction in induration of tuberculin reactions. The clotting times of these animals were markedly increased to 45 to 60 min.

Since changes in vascular permeability are associated with delayed reactions (8, 9) the effect of carrageenan on this factor was studied. Carrageenan-treated and control guinea pigs were given i.v. injections of Evans blue dye 12 hr after skin testing with O.T. 1:200, 1:500 and saline. In Table IV it may be seen that no significant differences were seen in the extent of blueing produced in the two groups despite the marked reduction in induration produced by carrageenan treatment.

Silica has similarly been reported to damage macrophages, so its effect on delayed sensitivity was also studied in a preliminary fashion. In a single experiment, guinea pigs injected with 20 mg of silica at the time of skin testing with 1:200 O.T. gave 24-hr reactions averaging 12 mm of erythema with reduced induration (+). This compared with control reactions of 20 mm of erythema and pronounced induration (+++).

DISCUSSION

A variety of treatments has been reported to inhibit the development of delayed hypersensitivity reactions. Suppression by methotrexate (10) and x-irradiation (11) may be the result of cell damage to a variety of mononuclear cells. Similarly vitamin A administration (12) or vitamin C depletion (13) produce damage by an effect on lysosomal enzymes of mononuclear cells. Antilymphocytic serum (14) may also produce a nonspecific depletion of mononuclear elements. In all of these there is some difficulty in determining whether damage to a single cell type is responsible for the suppression seen.

Since carrageenan and silica are more specifically lethal to macrophages (6) their suppres-

sive effect on delayed reactions is of some interest. In most instances their effect was primarily a reduction in the degree of induration and only with very large doses of carrageenan was the erythematous reaction eliminated. Histologic examination showed a marked decrease in the numbers of infiltrating mononuclear cells but without specific histochemical staining it could not be determined whether this reduction was primarily in macrophages. The greater persistence of erythema and vascular permeability, however, suggest that these later events occur independently of the bulk of the mononuclear infiltrate and may even be its cause.

Because carrageenans (particularly the λ variety, Viscarin) have been reported to have weak anti-coagulant properties (15) this possible mechanism of action took on considerable importance in view of the report of Cohen *et al.* (7) that the inhibitory effect of heparin and warfarin parallel their anticoagulant effect. In the experiments devised to deal with this point we found that all the carrageenans produced striking inhibition of tuberculin sensitivity in the face of normal clotting times. On the other hand, hirudin and heparin produced only mild to moderate inhibition with rather minor anticoagulation for the former agent, and pronounced anticoagulation with the latter. While it is certainly possible that an anticoagulant effect of carrageenan might be revealed by the use of a more sophisticated test of the clotting system, we feel that the present data strongly indicate that the inhibitory effect of carrageenan is neither dependent on nor related to anticoagulant properties.

There was some discrepancy between the results reported here with heparin and those of Cohen *et al.* (7). In our hands, heparin treatment produced marked decreases in erythema but less effect on induration. However, this may be only a matter of degree of sensitivity. In the experiments of Cohen *et al.* (7) guinea pigs were skin tested about 9 days after sensitization with either a contact sensitizer or a protein conjugate. Since our guinea pigs were tested at least 3 weeks after sensitization with mycobacteria, a much stronger degree of sensitivity would be expected and hence suppression would be more difficult to achieve. In this connection it might be noted that in the experiment reported in Table I in which guinea pigs had a high degree of tuberculin sensitivity, 100 mg of carrageenan were required to produce the degree of suppression achieved by 10-mg doses in other experiments.

While carrageenan has been reported to interfere with the complement system by inactivation of C′ 1 (16, 17) there is no evidence that its effect on delayed reactivity is mediated via this pathway. Complement levels in treated animals were not significantly altered under circumstances in which delayed reactions were markedly suppressed.

Studies from a number of laboratories on the mechanism of delayed hypersensitivity reactions have led to the conclusion that the first step in these processes is the interaction of antigen and a small number of specifically sensitized lymphocytes. The results of such immunologically specific reaction are presumably the release of soluble factors akin to or identical with the macrophage inhibiting factor found *in vitro* (18, 19). These lead to the secondary effects such as accumulation of immunologically nonspecific mononuclear cells and vascular permeability. Because this nonspecific mononuclear cell making up the bulk of the response has been shown to be a phagocytic cell derived from bone marrow (20) it is tempting to speculate that this is the target of action of such substances as carrageenan and silica.

Thus the common pathway shared by these substances is presumed to be their effect on the lysosomes of phagocytic cells, in particular macrophages (6). To the extent that these cells are important in the development of induration their inactivation might produce the observed effects. Similarly because these cells come from a continuously dividing cell population in the marrow (21) only a transient suppression might be expected, and in fact the effect of carrageenan injection was gone in 5 days.

The persistence of the erythema and vascular permeability in treated animals suggests that these are either direct results of the first step reaction of antigen with sensitized lymphocytes or that they are produced as a consequence of only minimal infiltration by the nonspecific mononuclear cells.

The primary observation reported here is that carrageenan and to a lesser extent silica, which are toxic for macrophages *in vitro*, produce a profound suppression in the induration seen in delayed sensitivity reactions *in vivo*. It seems reasonable to conclude, therefore, that macrophage-like cells make up the bulk of the indu-

tion normally seen and that these cells are the target for the effects of carrageenan and silica. Since the suppressive effects are temporary, it seems highly unlikely that the specifically sensitized lymphocytes are also affected.

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The Effect of Carrageenin on the Peptic Hydrolysis of Various Proteins

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Pepsin inhibition by carrageenin was studied to determine whether the addition of the polysaccharide to foods interferes with normal protein digestion. Different levels of carrageenin were added to solutions containing various concentrations of egg albumin, hemoglobin, casein, or soybean protein dissolved in HCl–citrate buffer, pH 1.6. Carrageenin at 0.085% level or lower did not affect pepsin activity in any of the solutions containing 1.0% or more protein, but there was marked inhibition of pepsin activity with higher concentrations of carrageenin or lower concentrations of protein. The protein level at which inhibition by a given concentration of carrageenin occurred, and the effect of pH on the degree of inhibition, varied from one protein substrate to another. Experiments in vivo further indicated that the levels of carrageenin used in foods do not interfere with normal protein digestion.

THE PROTEOLYTIC ACTION of pepsin may be inhibited by certain sulfated polysaccharides. In 1954, Levey and Sheinfeld (*1*) reported that heparin and chondroitin sulfate inhibit the pepsin-catalyzed hydrolysis of proteins, and more recently Houck *et al.* (*3*) reported that carrageenin, a sulfated polysaccharide from seaweed, is almost equally effective as an inhibitor of pepsin action. Data were obtained from studies both in vitro and in vivo suggesting that carrageenin affects pepsin activity. These authors reported that carrageenin ingested in drinking water (5 mg. per ml.) will inhibit ulcerogenesis in rats with pylorus ligation or subcutaneous injections of cortisone and in dogs subjected to large doses of histamine.

Since carrageenin is used as a stabilizer in many food products, the conditions under which pepsin inhibition by carrageenin occurs have been studied to determine whether addition of the polysaccharide to foods interferes with normal protein digestion. The results of these studies are presented.

Reagents and Methods

Casein, Hammerstein quality; soybean protein, 88% protein; and pepsin 5X Crystalline were obtained from Nutritional Biochemical Corp., and commercial grade carrageenin was obtained from Marine Colloids, Inc. Egg albumin was prepared from fresh hen eggs, and hemoglobin from fresh beef blood according to the procedure of Anson and Mirsky (*1*).

For tests in vitro, different levels of carrageenin were added to solutions containing various concentrations of egg albumin, hemoglobin, casein, or soybean protein dissolved in hydrochloric acid–citrate buffer, pH 1.6. The quantity of pepsin which was then added depended on the substrate and the assay used for following protein hydrolysis. Three different assays were employed, since no single procedure was suitable for measuring protein hydrolysis when different proteins are used as substrates. The Riggs and Stadie assay (*6*) was used with egg albumin, the Anson and Mirsky assay (*1*) with hemoglobin, and the Vollhard and Lohlein assay (*2*) with casein or soybean protein as the substrate.

For studies in vivo, male rats weighing approximately 150 grams were fasted 18 hours, and then given, by stomach tube, 2 ml. of a solution containing milk proteins with or without carrageenin. The dosing solutions were preparations of cow's milk modified to resemble human milk (carrageenin is used in most prepared infant formulas as a stabilizer). Exactly 1 hour after receiving the dosing solutions, the rats were killed with chloroform, the abdomen opened immediately, and the gastrointestinal tract ligated in two places: the esophagus directly above the stomach and the intestine 20 inches below the pylorus. The ligated segment was then removed, and the contents of the stomach and intestine were washed into a beaker with water. The

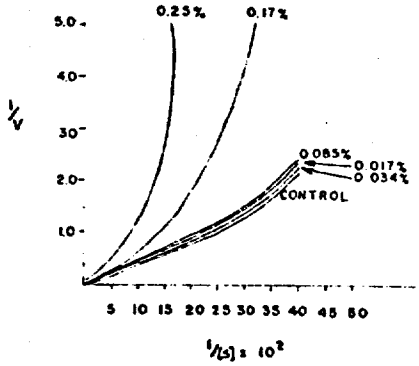

Figure 1. Effect of carrageenin on hydrolysis of egg albumin by pepsin

Egg albumin concentration [s], is expressed as mg. per ml. of incubation mixture, and velocity, v, is expressed in arbitrary units

Table I. Effect of Carrageenin on Digestion of Milk Proteins in vivo[a]

Rat Weight,[b] grams	Carrageenin Concn., %	Protein Digested,[c] mg.
144 ± 17	0.000	44.3 ± 8.6
145 ± 24	0.017	50.4 ± 5.4
151 ± 36	0.175	41.9 ± 5.4
147 ± 8	0.525	36.6 ± 5.8

[a] 2 ml. of a cow's milk formula modified to resemble human milk was given each rat by stomach tube. Formulas contained 1.72% protein. Carrageenin was added to formulas at levels shown in table.

[b] Av. weight (six rats per treatment) ± std. dev.

[c] Av. mg. of protein ± std. dev. digested during a 1-hour period.

mixture was brought to boiling temperature very rapidly. The protein in the boiled solution was precipitated with 4% trichloroacetic acid and the nitrogen content of the precipitate determined by the semimicro-Kjeldahl method. The difference between the protein—grams of nitrogen multiplied by 6.38—in the test solutions before ingestion, and the amount found in the stomach and upper intestine was considered to be the amount of protein digested in one hour.

Results

Egg albumin was incubated with pepsin (100 μg. per ml.) for four minutes at pH 1.6 and 30° C. Preliminary experiments showed that the rate of hydrolysis of egg albumin was proportional to the pepsin concentration in the range 0 to 100 μg. per ml. and that the reaction rate remained constant for at least 10 minutes. The effect of various concentrations of carrageenin on pepsin activity is shown in Figure 1 as a Lineweaver-Burk plot. Carrageenin at 0.25% or lower did not affect pepsin activity in any of the solutions containing 2.5% ($1/[s] \times 10^2 = 5$) or more of egg albumin, but there was inhibition in all solutions containing less than 0.5% protein and 0.17% or more carrageenin. When the level of carrageenin was below 0.085%, there was no inhibition of pepsin activity even at very low concentrations of protein (0.25%). The data obtained with hemoglobin as substrate were similar to the data with egg albumin shown in Figure 1.

When soybean protein or casein was the substrate, a concentration of 0.5 mg. of pepsin per ml. of solution was incubated with the substrate for 15 minutes at pH 1.6 and 30° C. The effect of carrageenin on pepsin activity with casein as substrate is shown in Figure 2. There was little inhibition by carrageenin at any concentration studied when 1.0% or more protein was present in the incubation mixture.

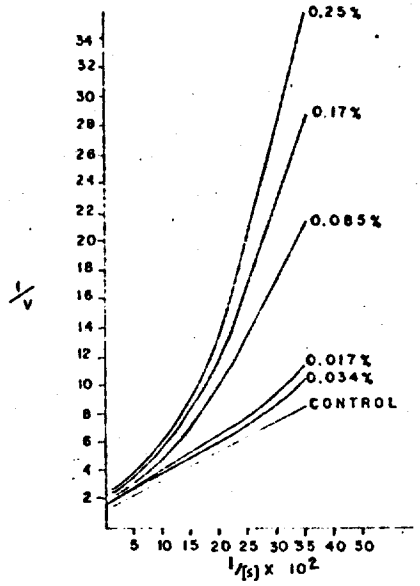

Figure 2. Effect of carrageenin on hydrolysis of casein by pepsin

Casein concentration [s], is expressed as mg. per ml. of incubation mixture, and velocity, v, is expressed in arbitrary units

Likewise, there was little or no inhibition by 0.034% or less of carrageenin at any concentration of protein studied (0.3% or more). The results obtained with soybean protein were almost identical to the results with casein shown in Figure 2.

The effect of carrageenin on the pepsin-catalyzed hydrolysis of casein was further studied by measuring pepsin activity in the presence or absence of carrageenin at pH values ranging from 1 through 7. Initially, the incubation solutions contained 1.329 mg. of casein, 0.5 mg. of pepsin, and 0.0 or 0.85 mg. of carrageenin per ml.; pepsin activity is inhibited at these concentrations of casein and at 0.85 mg. of carrageenin per ml. The solutions were incubated for 15 minutes at 40° C., and the desired pH was maintained in a given incubation mixture by continuous titration with dilute hydrochloric acid or sodium hydroxide solution. Pepsin activity measurements were not made in the pH range 4.1 to 5.9, since casein is only very slightly soluble in water at these concentrations of hydrogen ions.

Pepsin activity (mg. of amino nitrogen × 10) in the presence or absence of carrageenin with casein as substrate is shown in Figure 3 as a function of pH. Pepsin activity reached a maximum around pH 2.5 and rapidly decreased as the solutions were made more alkaline. The high (0.085%) concentration of carrageenin inhibited pepsin activity at all pH values from 1 through 7, but the degree of inhibition was less at values above 2.

The effect of pH on pepsin activity in the presence or absence of carrageenin was also studied using hemoglobin as a

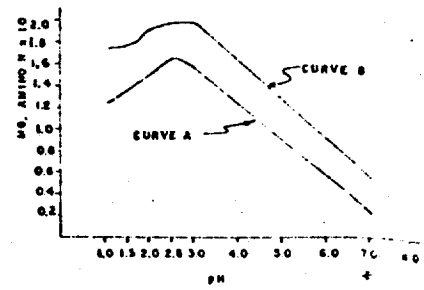

Figure 3. Effect of pH on hydrolysis of casein by pepsin in the presence (curve A) or absence (Curve B) of carrageenin

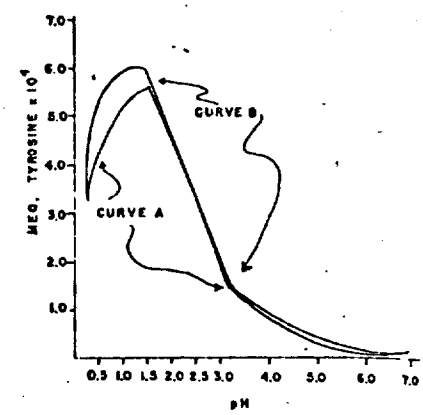

Figure 4. Effect of pH on hydrolysis of hemoglobin by pepsin in the presence (Curve A) or absence (Curve B) of carrageenin

substrate. In these experiments, the incubation solutions contained 2.24 mg. of hemoglobin, 0.01 mg. of pepsin, and 0.0 or 0.85 mg. of carrageenin per ml. The solutions were incubated for 15 minutes at 25° C. The results, expressed graphically in Figure 4, indicate that carrageenin does not inhibit the peptic hydrolysis of hemoglobin at pH values greater than 2.

The data in Table I indicate that protein digestion proceeded normally in rats fed 2 ml. of test formulas simulating human milk which contained 1.72% protein and from 0 to 0.175% carrageenin. None of these differences were statistically significant. The 36.6 mg. of protein digested by rats receiving a formula with 0.525% carrageenin was, however, significantly ($P = 0.05$) lower than the other groups. Formulas containing a carrageenin level greater than 0.525% were not tested, since such solutions are extremely viscous and will not flow through a stomach tube under the conditions of the procedures used with solutions containing less carrageenin.

Conclusions

It is concluded from the experiments in vitro that carrageenin is a competitive inhibitor of pepsin activity—the in-

hibiting ability depends on the concentration of protein substrate relative to the concentration of carrageenin—and that the degree and character of this inhibition depends on the particular substrate used in the assay for enzymatic activity. The data support the conclusion of Piper and Fenton (5) that carrageenin does not react with the active center of the enzyme but inhibits by combining with the substrate. The carrageenin in food products should not interfere with normal peptic digestion of protein, since the concentration of protein is higher and the concentration of carrageenin much lower in such products than in the experimental mixtures where inhibition was demonstrated. Although the exact concentration of protein necessary to prevent inhibition of pepsin by a given concentration of carrageenin depends on the kind of proteins in the mixture, a low concentration (0.25%) of any dietary protein would probably be sufficient at the carrageenin levels used in food products. The conclusions drawn from the experiments in vitro are supported by the data from animal feeding experiments summarized in Table I.

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SHORT COMMUNICATION

Ulcerative colitis in the guinea-pig caused by seaweed extract

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Carrageenans are widely used as food ingredients as well as medicinally in various parts of the world (Bonfils, 1968). As yet there have been no reports of adverse effects following the use of these substances (Anderson, 1967).

Recently, we have found a high incidence of ulcerative colitis in several species of laboratory animals fed extracts of various red seaweeds (Marcus & Watt, 1969).

In this paper we present our findings on degraded and undegraded carrageenans derived from *Eucheuma spinosum* and fed to guinea-pigs over a relatively short time.

Method

Adult male albino guinea-pigs, of 500 g average body weight, bred in our own laboratory, were housed in separate cages and fed a normal cube diet (S.G.1) supplemented daily with fresh cabbage.

One group of 10 animals received as drinking fluid a 1% aqueous solution of undegraded carrageenan[1] derived from *E. spinosum*. A second group of 10 animals received as drinking fluid a 5% aqueous solution of degraded carrageenan[2] also derived from *E. spinosum*. The degraded product, being less viscous, was used at a higher concentration than the undegraded product. Solutions of carrageenan were freshly prepared each day, stored at 4° and supplied in drinking bottles that were cleaned daily; no gross bacterial contamination was observed. Allowing for spillage from the drinking bottles, the daily intake per animal of undegraded carrageenan was not more than 1·5 g/kg and of degraded carrageenan not more than 2 g/kg body weight. A third control group of 10 guinea-pigs received water *ad lib* but without added carrageenan.

At frequent intervals throughout the experiment, the stools were examined for occult blood using the Hematest method. The animals were killed, using ether anaesthesia, between 20 and 30 days after the start of the experiment. The gastro-intestinal tract was entirely removed. The large bowel was emptied of faeces, distended with 10% formol saline, and after fixation, carefully examined. Histological sections were prepared from various parts of the colon and stained with haematoxylin and eosin, and also with toluidine blue.

Results

In the first group which received undegraded carrageenan, four guinea-pigs were killed after 20 days and two of these showed multiple ulcerative lesions in the caecum. The remaining six animals were killed after 30 days; traces of occult blood in the faeces were present from day 23. These animals had ulcerative lesions in the caecum

[1] Undergraded carrageenan was obtained from J. W. Cumming & Son Ltd, Salford.
[2] Degraded carrageenan was kindly supplied by Laboratories Glaxo-Evans, Paris.

and two had lesions extending into the colon for a short distance. The overall incidence of ulceration in this group was 80%.

In the second group, which received degraded carrageenan, most of the animals showed looseness of the stools by the end of 10 days; from the 20th to the 30th days, all had occult blood in the faeces. In this group the incidence of ulcerative colitis was 100%. In five animals killed between the 20th and 25th days, the lesions were mainly in the caecum; in the remainder, killed between the 26th and 30th days, ulceration had extended into the lower colon and rectum.

In both experimental groups the lesions were multiple, numbering sometimes in the hundreds. Macroscopically, they consisted of pin-head sized ulcers, rounded or slightly irregular in outline. Some had coalesced to form larger lesions, often linear in shape. Histologically, the ulcerations involved mainly the mucosa and showed features of both acute and subacute inflammatory infiltration, as well as crypt abscesses (Fig. 1). Macrophages containing metachromatic material were frequently seen in toluidine blue stained sections.

In the control group, the animals remained healthy throughout the 30 days. There was no occult blood in the stools and no pathology in the caecum, colon or rectum.

FIG. 1. Subacute ulcer in colon of a guinea-pig fed carrageenan: heavy cellular infiltrate in ulcer base and in submucosa. H. & E. × 56.

Discussion

Our results have revealed a hitherto undescribed effect of carrageenan, namely ulceration of the caecum and colon in the guinea-pig. The lesions produced are not readily visible unless the bowel is emptied of faeces and examined using transmitted light. This is perhaps why such lesions have not previously been observed.

The more severe ulceration in the animals on degraded carrageenan is more likely to be due to the greater total amount of carrageenan consumed by these animals than to possible contaminants arising as a result of the degradation process.

The ulcerative lesions in the guinea-pig are not identical in distribution with those typically seen in ulcerative colitis in man. Nevertheless, their histological appearance and their confinement largely to the mucosa are features common to both. The significance of our results in relation to human ulcerative colitis is at present only speculative and must await more comprehensive investigation.

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Ulcerative colitis in rabbits fed degraded carrageenan

We recently described the occurrence of ulcerative colitis in guinea-pigs fed orally in their drinking water degraded and undegraded carrageenan derived from the red seaweed *Eucheuma spinosum* (Watt & Marcus, 1969). The lesions produced by degraded carrageenan were severe and involved the whole length of the colon.

We have found that degraded carrageenan derived from the same seaweed also causes severe damage to the colon of the rabbit. In this communication we report on the incidence of ulcerative lesions found in rabbits fed various concentrations of the degraded product.

Twenty male Californian rabbits of 2950 g average body weight were housed in separate cages and fed a standard cube diet (S.G.1). Three experimental groups, 5 rabbits in each group, received as drinking fluid 5, 1 and 0·1% respectively aqueous solutions of degraded carrageenan derived from *Eucheuma spinosum*.* The solutions of degraded carrageenan were freshly prepared daily, stored at 4°, and supplied *ad lib* in drinking bottles which were cleaned each day. The volume of fluid consumed per animal per day was measured throughout the 6 to 12 week period of the experiment. Control animals received water *ad lib* but without added carrageenan. At weekly intervals, the animals were weighed and their faeces examined for occult blood using the Haematest method. At the end of the experiment, the animals were killed with pentobarbitone. At post-mortem examination, the colon was removed, emptied of faeces and examined for the presence of ulcerative lesions.

Animals fed degraded carrageenan at the 5% concentration in their drinking water received on average a daily dose of 1·4 g/kg weight over a 6 week period. Diarrhoea associated with visible and occult blood in the faeces developed by the end of 7 days and persisted. The animals rapidly lost weight, the average loss at the end of the

experiment being 976 g. All animals in this group showed severe ulceration of the colon.

Animals fed degraded carrageenan at the 1% concentration received on average a daily dose of 0·8 g/kg weight over a 7 week period. Only 1 animal developed diarrhoea; occult blood was present in the faeces in all of the animals after 2 weeks. The average weight loss in the group was 190 g. All of the animals showed ulceration of the colon of moderate severity.

Animals fed degraded carrageenan at the 0·1% concentration received on average a daily dose of 0·07 g/kg over a 12 week period. Diarrhoea did not occur but occult blood in the faeces was demonstrable in 3 of the animals by the end of 10 weeks. One animal lost 30 g in weight; the remainder gained weight, the average weight gain in the group being 238 g. Multiple ulcers in the colon were found in 3 of the 5 rabbits.

Control animals drank on average 370 ml water per day over a 12 week period. There was no diarrhoea or occult blood in the faeces at any time. The average gain was 1218 g. No ulcerative lesions were found in the colon in any of the control animals.

The results indicate that degraded carrageenan at doses of 0·07 g/kg per day will produce ulcerative lesions in the colon in 3 out of 5 rabbits when given orally in the drinking fluid over a period of 3 months. It is possible that an even higher incidence of lesions may have been found had the feeding experiment been prolonged.

In this country, degraded carrageenan derived from *Eucheuma spinosum* has been used only in clinical trials in the treatment of chronic peptic ulcer (Evans, Lowell & Thomas, 1965). On the continent, however, the product in tablet form is widely used to reduce peptic activity and gastric acidity in gastro-duodenal disorders (Bonfils, 1968). The maximal dose recommended per day is about the same (0·07 g/kg) as produces ulcerative lesions in the colon of the rabbit.

In the light of these findings, together with our results in three other animal species (Marcus & Watt, 1969), it seems that the continued use of degraded carrageenan as a drug in the treatment of peptic ulcer may not be without risk.

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October 17, 1969

* Carrageenan degraded by mild acid hydrolysis and retaining about 29% sulphate was kindly supplied by Laboratoires Glaxo, Paris.

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HYPERPLASTIC MUCOSAL CHANGES IN THE RABBIT COLON PRODUCED BY DEGRADED CARRAGEENIN

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Degraded carrageenin derived from the red seaweed *Eucheuma spinosum* was fed to rabbits in their drinking water over a period of 3 months. In one group in which the average daily dose per animal was 0.8 g per kg of body weight, all 6 animals showed ulcers of the colon; in 3, there were well marked hyperplastic mucosal changes including pseudopolyps and polypoidal lesions of glandular type. In a second group in which the average daily dose per animal was 0.07 g per kg, 3 of the 6 rabbits showed ulcers in the colon; hyperplastic mucosal changes were observed on microscopic examination only. The use of carrageenin as a therapeutic agent or as a food additive will have to be reconsidered.

We recently reported the occurrence of ulcers in the colon in various laboratory animals fed carrageenin.[1] In the rabbit, degraded carrageenin derived from the red seaweed *Eucheuma spinosum* can produce ulceration of the colon when fed in the drinking water at low concentrations.[2] The lesions can involve the whole of the colon including the rectum.

We are at present investigating the effects of prolonged administration of carrageenin, in particular, the occurrence of hyperplastic changes and the possibility of malignant transformation of the colonic mucosa, a well recognized hazard of chronic ulcerative colitis in man. In this communication, we describe the results of experiments in which rabbits were fed degraded carrageenin over a period of 3 months.

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The authors thank Professor Donald A. Heath for his helpful criticisms of the manuscript and Mr. D. R. Williams for the photography. Degraded carrageenin was kindly supplied by Laboratoires-Glaxo, Paris, France.

Materials and Methods

A total of 18 young adult male California rabbits of 2700 g average body weight were housed in separate cages and fed a standard cube diet (S.G.1). Experimental rabbits (two groups of 6 animals) were supplied each day with a freshly prepared solution of drinking water containing degraded carrageenin. The carrageenin was derived from the red seaweed *E. spinosum* and had been degraded by mild acid hydrolysis so as to retain about 29% sulfate content. A control group of 6 animals received water without added degraded carrageenin.

The initial and weekly weights of all animals were recorded, and tests for occult blood in the feces were carried out with the use of the Haematest method. At the end of 3 months, the two experimental groups were killed to study the effect of degraded carrageenin on the colon. One group had received a 1% aqueous solution of degraded carrageenin, the other a 0.1% solution. The average daily dose per animal in the two groups was 0.8 g per kg and 0.07 g per kg of body weight, respectively.

The colons were removed, washed, and fixed in formol-saline. They were examined by direct and transmitted lighting. Sections for histological examination were stained with hematoxylin and eosin, and also with toluidine blue. The colons from control animals were similarly treated.

Results

Loss of weight occurred in all of the animals fed the 1% aqueous solution of degraded carrageenin, the average loss in the group after 3 months being 220 g. Animals on the 0.1% aqueous solution maintained their weight or showed a slight gain (average 270 g); this was small in comparison with the control group receiving water without degraded carrageenin (average gain 1590 g). One animal receiving the higher concentration of degraded carrageenin developed persistent diarrhea; 2 animals in the same group showed a mild transient diarrhea on several occasions during the first 2 months. No diarrhea occurred in the animals receiving the lower concentration of degraded carrageenin. Occult blood was demonstrable in the feces in 9 of the 12 animals in the experimental groups by the end of the 3-month period.

All of the rabbits fed degraded carrageenin in their drinking fluid at the 1% level showed ulceration of the colon. In the group receiving degraded carrageenin at the 0.1% level, ulcers were present in 3 of the 6 animals. The ulcers occurred mainly in the cecum, but also in the proximal colon in the animals receiving the higher dose of degraded carrageenin. Ulcers situated on the summits of the transverse folds were linear in type; those in the interfold areas were round or irregular in outline and measured up to 1 cm in diameter (fig. 1). On microscopic examination, the ulcers were mostly confined to the mucosa; in occasional lesions, the muscularis mucosae was breached (fig. 2). The ulcer base showed inflammatory cell infiltration as well as fibroblastic proliferation. The cellular infiltrate was comprised of polymorphonuclear leukocytes, lymphocytes, plasma cells, and macro-

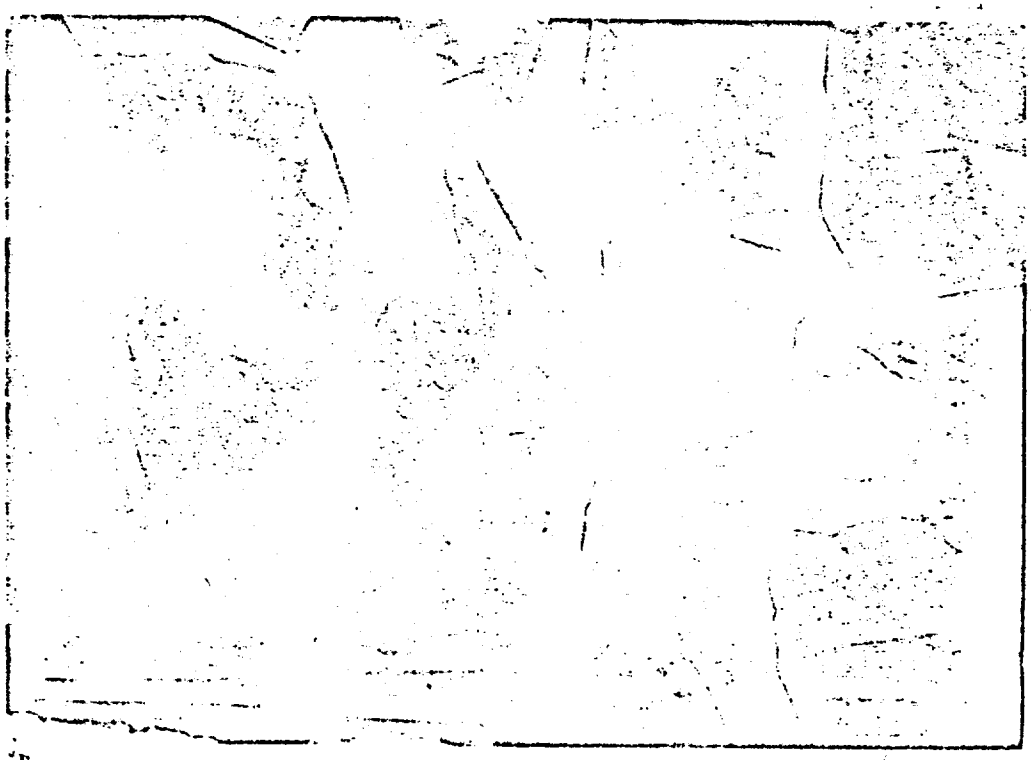

FIG. 1. Colon of rabbit fed 1% aqueous solution of degraded carrageenin over a 3-month period. Multiple ulcers as viewed by transmitted light. The ulcers are irregular in outline, involve both the folds and the haustra, and are surrounded by a thickened mucosa (× 2.3).

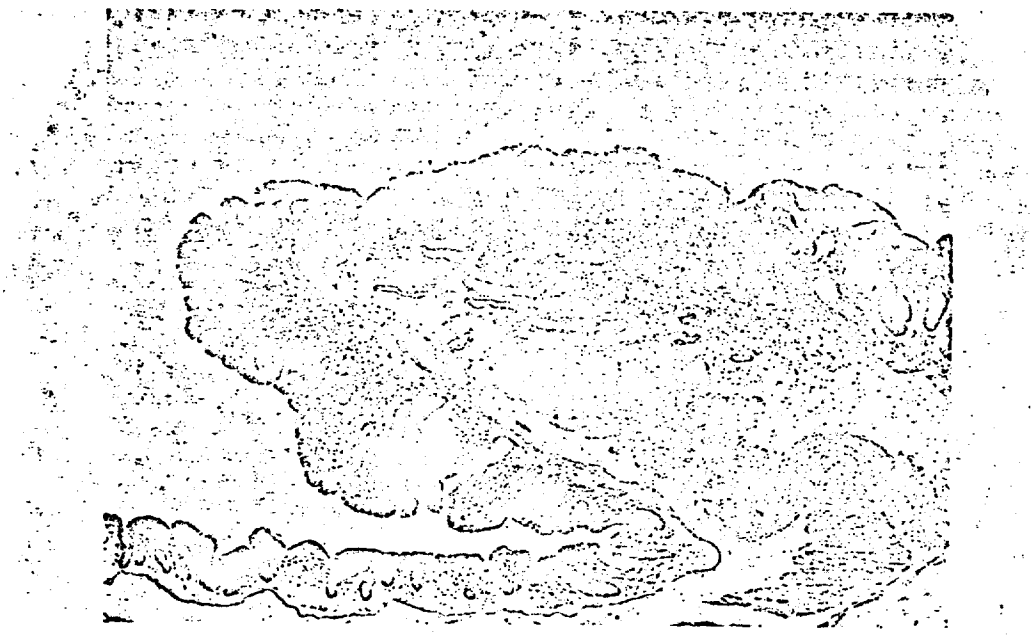

FIG. 2. Ulcer on one side of a transverse fold of the cecum. The muscularis mucosae has been destroyed. The ulcer base shows acute and chronic inflammatory cell infiltration as well as fibroblastic proliferation (hematoxylin and eosin, × 35).

phages, often containing material which was metachromatic in toluidine blue-stained sections. Although there were no crypt abscesses, microabscesses were occasionally present in the mucosa at the ulcer margins (fig. 3).

Hyperplastic mucosal changes

Gross thickening of the mucosa was readily visible macroscopically in 3 of the 6 animals fed the 1% aqueous solution of degraded carrageenin. The mucosal thickening presented as small, sessile, polypoidal formations measuring up to 0.3 cm in diameter and in height. They were present mainly in the cecum and were situated on the transverse folds or in the haustra between the folds (figs. 4 and 5). They frequently occurred at the margins of ulcerative lesions but were also found independently well beyond the ulcer margins. In 2 of these animals, the polypoidal formations were so striking as to be the dominant pathological feature.

On microscopic examination the polypoidal formations were of two types.

Pseudopolyps. These consisted of edematous congested mucosa, often infiltrated with acute inflammatory cells and occurring particularly at ulcer margins.

Glandular polyps. These consisted of markedly thickened mucosa in which there was pronounced hyperplasia of the mucosal glands, in association with chronic inflammatory cell infiltration of varying degree (figs. 6 and 7). Small villous projections from the surface or lateral aspects of such lesions were occasionally observed. The epithelium of the glandular acini was often multilayered and mitoses were frequent. Compared with nonhyperplastic glands, both in experimental and control groups, there was a 2- to 3-fold increase in the number of cells showing mitotic activity. The hyperplastic epithelial cells were regularly arranged and showed no evidence of malignancy (fig. 8). The mucosa of the colon elsewhere was generally thicker than normal and showed areas of glandular hyperplasia and dilatation, as well as pseudopolypoid formations similar to those described above but smaller. Chronic inflammatory cell infiltration was also present.

In the animals fed the 0.1% aqueous solution of degraded carrageenin, the

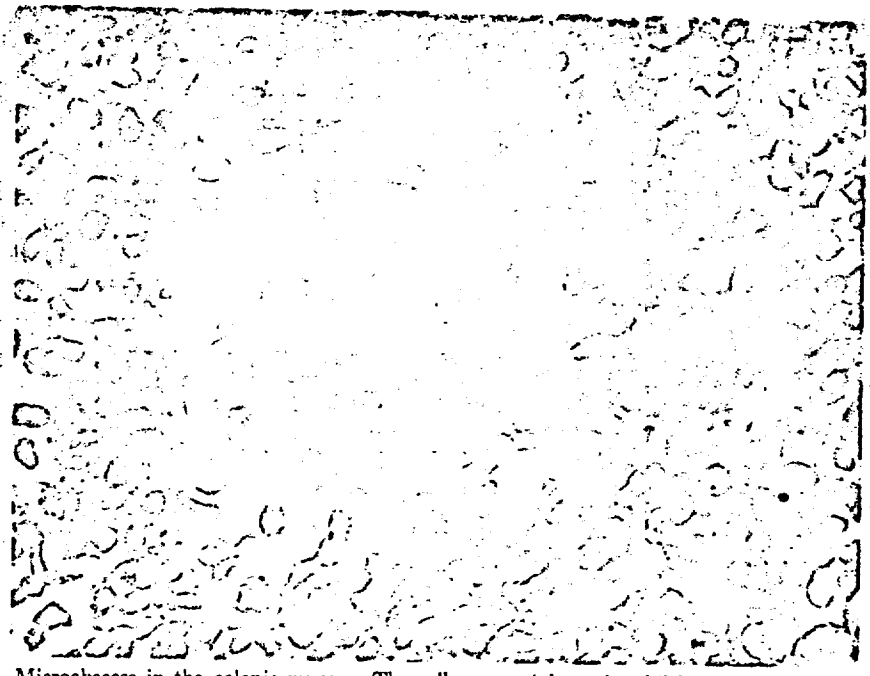

FIG. 3. Microabscess in the colonic mucosa. The cells are mainly eosinophil leukocytes (hematoxylin and eosin, × 600).

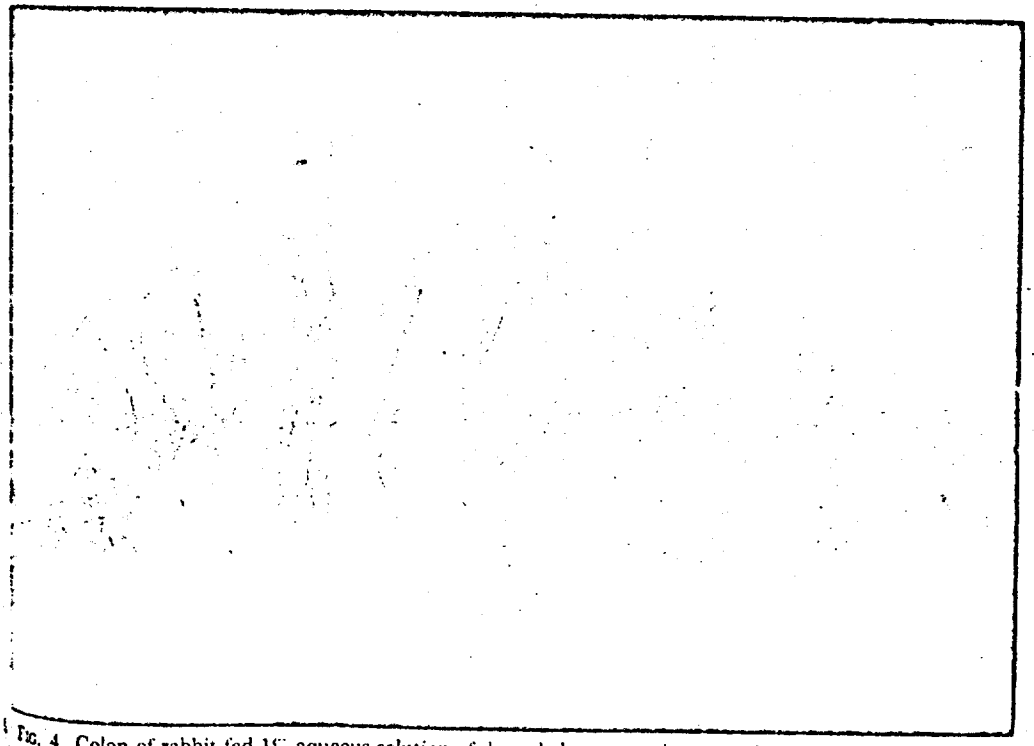

FIG. 4. Colon of rabbit fed 1% aqueous solution of degraded carrageenin over a 3-month period. Polypoidal ulcerations on the cecal folds and in the haustra (× 0.7).

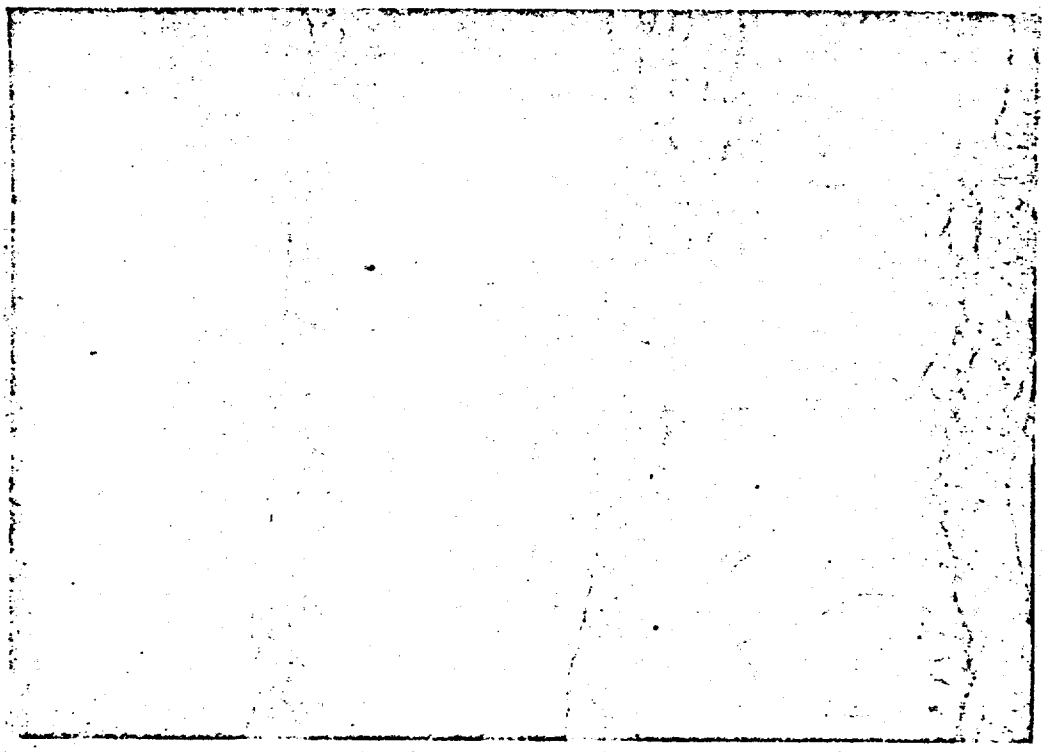

FIG. 5. Close up view of polypoidal formations in the haustra alongside the folds (× 2.5).

FIG. 6. Colon of rabbit fed 1% aqueous solution of degraded carrageenin. Polypoid lesion showing marked hyperplasia of the mucosal glands, villous structures on surface and lateral aspects, and chronic inflammatory cell infiltration of stroma (hematoxylin and eosin, × 48).

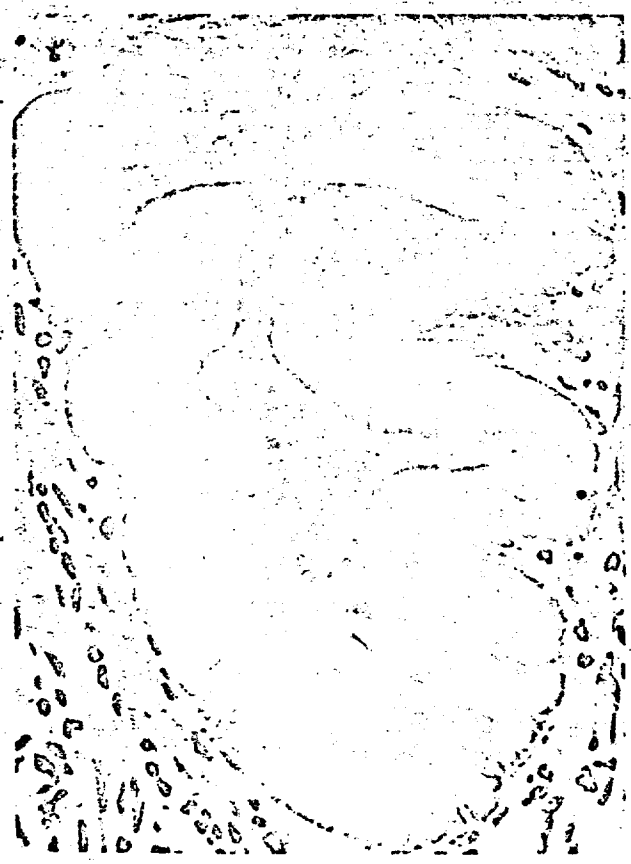

FIG. 8. Hyperplastic mucosal gland showing multilayered epithelium and at least six mitotic figures; cells regularly arranged; no evidence of atypia (hematoxylin and eosin, × 375).

polyps of a similar kind are often seen in ulcerative colitis in man and are no longer considered to be precancerous.[3-5]

Some of the glandular polypoidal formations in our experimental animals occurred in close proximity to ulcerated areas and can perhaps be dismissed as a manifestation of reactive hyperplasia. Other polypoidal formations were observed in the haustra and were not related to the margins of ulcerated lesions. Originally in these areas, there may have been acute superficial ulceration which had subsequently healed, leaving, as might be expected, no histological evidence of ulceration. Alternatively, these hyperplastic lesions may have arisen per se in the absence of any associated ulcerative process. Microscopically, they were mainly glandular in pattern, although some showed definite villous structure on the surface or lateral aspects.

In rabbits fed degraded carrageenin over a 3-month period, these hyperplastic glandular polypoidal formations have shown no evidence of precancerous change. Long term investigations, however, are imperative in order to exclude this possibility.

Degraded carrageenin derived from *E. spinosum* has been used medicinally in the United Kingdom only in clinical trials,[6] but is widely used in tablet form (Ebimar, Glaxo-Evans, Paris) on the continent as a pepsin inhibitor in the treatment of gastroduodenal disorders, particularly peptic ulcer.[7] The maximal recommended dose per day in man is of the same order (0.07 g per kg of body weight) as produces hyperplastic and ulcerative

FIG. 9. Colon of rabbit fed 0.1% aqueous solution of degraded carrageenin over a 3-month period. Microscopic pseudopolypoidal formation showing edema of the stroma and infiltration by inflammatory cells but without hyperplasia of glands. The mucosa on the right is slightly thicker than normal and is also infiltrated (hematoxylin and eosin, × 48).

FIG. 10. Colon of rabbit fed 0.1% aqueous solution of degraded carrageenin. The mucosa is thickened because of chronic inflammatory cell infiltration and well marked glandular hyperplasia (hematoxylin and eosin, × 48).

lesions in the colon of the rabbit. It is evident that the continued use of this degraded product is not without risk.

Carrageenin in its undegraded form and derived from various seaweeds, including *E. spinosum*, is incorporated in a wide variety of foodstuffs in many parts of the world including the United Kingdom. At percentage levels which range up to 1%, it is contained in blancmanges, chocolate products, chocolate-flavored drinks, chocolate milk, pressure-dispensed whipped cream, evaporated milk, cheese spreads and cheese foods, ice cream, frozen custard, sherbets, ices, French dressing and salad dressing, artificially sweetened jellies and jams, milk puddings, pie fillings, dietetic foods, beer, soups, sauces, soft drinks, fruit drinks, syrups, toppings, baked goods including bread doughs and mixes, and even in toothpaste—a list which is by no means exhaustive. Either in the process of preparation of such foods or during passage through the stomach or small intestine, it is probable that some degree of degradation of the carrageenin takes place. Although the cause of ulcerative colitis in man is as yet unknown, it is possible that, in some cases at least, the intake of carrageenin-containing foods may be an important etiological factor.

As recently as June 1969, the World Health Organization gave a clean bill of health to the use of carrageenin as a food additive. However, in view of our reported findings in four animal species[1] and particularly in the rabbit, in which ulceration of the colon and associated hyperplastic mucosal changes can be induced by feeding relatively small amounts of degraded carrageenin, it is evident that the use of carrageenin either as a food additive or as a drug will have to be reconsidered.

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HISTOCHEMICAL AND HISTOENZYMOLOGICAL RESEARCH ON CARRAGEEN D. B. [1] AT THE GASTRIC MUCOSA

by

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INTRODUCTION

The protection of the gastric mucosa against aggressive agents both external (alcohol, foods which are too acid or too alkaline, biochemical or pharmacologica changes in the gastric secretion such as (illegible) , cortisone , ACTH, and internal (effects of physical stress, prolonged fasting) presents serious biological problems both for the clinician and the researcher because of the lack of knowledge on the subject of the actual determinism of the pathogeny of ulcers and certain gastrites.

Although it has been more or less established that the production of ulcers can occur even on the atrophic mucosa , it seems that its appearance is not attributed to the rate of chlorhydric secretion (Baker and Bridges, 1954), but rather to an increase in the peptic activity of the gastric mucosa (Bonfils and Lambling, 1960). It seems, however, that besides this factor, there are a certain number of other elements which intervene and, in particular, changes in the capillary vascularization of the chorion and in the permeability of the basal membranes of the glandular tubes. That these perturbations are due to hormonal, allergic, cortico-viscero-humoral or vascular effects does not at all change the fact that in the last analysis (illegible) leads to complex modifications of the gastric chemistry and a characteristic morphological (illegible) of ulcers. Also we must not confuse as being the same entity an experimental ulcer and the ulcer sickness.

Numerous protection methods have been proposed aiming at essentially correcting the major trouble, that of the illegible hypersecretion and reinforcing the muco-secretive power of the mucosa, consequently illegible the equilibrium between the defenses of this mucosa and the illegible factors of the ulcerogenic aggression. Effort was aimed therefore at reestablishing the mucous film, the mucin which is capable of opposing both the chlorhydric secfetion and the peptic secretion.

One of the means consists in coating the epithelial surface with bismuth salts which adhere closely to the damaged tissues and favoring the epithelization of the ulcers. The quantity of bismuth which thus adheres to the walls of the ulcers varies according to the type of bismuth and the substances which are associated to it (Feinblatt, 1960; La Barre, 1961; Roi, 1954) as shown by the bismuth content of the dry residue of the stomach after incineration according to the method of Chefney. Furthermore it seems that the bismuth acts rather on the reduction of the free acidity, although there does not seem to be any relationship between the rate of the free acid and the appearance or non-appearance of ulcers. But the bismuth also seems to act on ulcers of different etiologies.

It has been established that the appearance of severe lesions of the mucosa

in the rat is attributable to the nature of the ulcerogenic determinant or cause. Reserpin at 250γ for 5 days causes ulcers limited to the region of the antrum and Shay's method (Peremans, 1957) , on rats deprived of food fof 30 hours followed by a ligature of the pylorus for 2 to 24 hours, of ulcers localized at the rumen (paunch) of the stomach. Ulcers by administration of histamine in oily solution in the rat and by instillation of chlorhydric acid 3.8% twice a day for 10 days in the guinea pig, according to the method of Mann, has no fixed location but are situated primarily in the region of the small curvature of the pylorus.

A second group of protective agents concerns the use of gastroduodenal extracts tending to increase the release of mucoproteose in the stomach, this mucoproteose being the product of the destruction of the mucus of the surface epithelium of the gastric mucosa (Schmid, 1951). Unfortunately they have a tendency to inhibit the gastric motricity although they do not contain enterogastrone. The inhibiting substances of the gastric secretion which they seem to contain are not known.

These two types of empirical protection make it possible to understand the reasons which have brought pharmacologists to use substances of a nature more directly related to the mucous secretion and which we could call "mucomimetic".

The mucin includes an active component, the sulphuric mucoitin acid, capable of opposing an inhibiting power to the proteolytic power of the pepsin and, furthermore, possesses a buffer power, neutralizing the excess in acid secretion by means of its bicarbonates. Its protective properties with regard to the acid ulcerogenic aggression are experimentally revealed by the fact that a mechanical traumatism of a gastric sector deprived of mucin causes the formation of a prolonged ulcer. The same traumatism on a mucous membrane covered with mucin is without effect. Furthermore, the administration of mucin to animals having cortisonic ulcers causes a rapid resprption of the ulcerous lesions and a reduction in the rate of the pepsin.

The mechanism of action is to be sought in the tendency which the polysaccharides have of combining with proteins; the sulphuric mucoitin acid, bound with pepsin, constitutes an inactive group from the enzymatic point of view on which the chlorhydric acid has no effect. It therefore seemed logical to seek substances presenting the same characteristics as the mucin to replace mucin with them in anti-ulcer therapy.

Babkin and Komarov (1932), Zaus and Fosdick (1934), Levey and Scheinfeld (1954) and Placer, Roubal and Volkac (1958) published papers on the various sulfated polysaccharides of biological origin such as heparin and sulfuric chondroitin acid and propose to use them because of their antipeptic properties. Rosen et al. (1956) used, in addition to the latter, other sulfates of polysaccharides such as polyanhydromannuronic acid.

Bonfils and Lambling (1960), comparing the activity of various polysaccharides, obsdrved that heparin β is the most active in vitro from the point of view of protection against the proteolytic activity of the pepsin, followed by polysaccharide C_{16}, sulfuric chondroitin acid and lastly heparin α ; in vivo the order is different: heparine alpha, heparin beta, polysaccharide C_{16}, sulfuric chondroitin acid.

This is why in current therapy preference is given to two polysaccharides extracts of Chondrus crispus: the polysaccharide C_{16} which is a partially degraded product and carrageen (carraghenine) DB which is the natural non-degraded product, the antipeptic activity of which was studied by Peindaries in 1964, in comparison

with the carbonate and sub-nitrate of bismuth. According to this author the carrageen DB, a natural non-degraded product, would have the greatest antipeptic power. In any event, in vivo in the animal which pylorum bound, the antipeptic effect of these two substances is much more distinct and more intense than that of the bismuthed derivatives. They protect the mucous (membrane) against the appearance of ulcers, without changing the gastric secretion which occurs regularly.

In morphological terms it can be considered that the extract of carrageen DB has an antizymogenic effect and we can predict its action on the principal cells and on the mucous membrane to which it would substitute itself to neutralize the zymogen and to form with it a compound of prolonged action.

The pepsinogen secreted in the inactive form is not charged. Its activity results from the appearance of an amphoteric charge and the pepsin has, in an acid medium, a positive charge. The polysaccharide, in its turn, in acid medium is a poly-anion of negative charge. The inactive compound resulting therefrom would explain the antipeptic power.

We have therefore attempted to analyze, by sufficiently sensitive methods, these effects, by interrogating all of the cells implied in this secretion. These investigations consist above all in the study of a certain number of histoenzymological and histochemical activities capable of translating relations of cause to effect.

METHODOLOGY

The investigations were carried out in two parts. In the first part we first of all examined the physiological changes in the normal animals , then we subjected them to various aggressions by alcohol, chlorhydric acid, histamin, by an antihistamine followed by the effect of the histamine. A group of animals received a dose of carrageen DB proportional to the aggressor effect and we followed the phenomena of correction. This orientational research served as a basis to us for the second part of our work during the course of which we subjected the animals to an extended fasting under conditions very close to the production of experimental ulcer according to Shay's method, but without ligature of the pylorum. On the other hand, during that period we subjected them to psychic stress. Another group was fed normally while subjecting it to psychic stress.

In both cases prior to the sactifice we administered carrageen DB and we examine the appearance of the gastric mucosa.

First part

The first part of the experimentation was done on 50 adult Wistar male rats weighing from 160 to 240 grams. The animals all came from the same breeding farm and received a standard balance diet and water as much as desired.

The animals were broken down into two batches:
A. control batches or groups
1. a first control batch in which the subjects served for the establishment of the enzymatic "chart" of the stomach. In the analysis of the mucosa there were included the physiological changes in the nyctohemeral cycle. These data are considered in all of the rest of the experimentation.

2. A second control batch received the carrageen DB in solution at 1% in water. The product was administered by souple oesopagean probe in order to prevent any mechanical irritation. The doses of the carrageen DB administered were between 0.2 and 1 ml of total volume, which corresponds per animal to quantities on the order of 0.125 to 0.416 ml for 100 g of weight. The actual quantity given each animal varied therefore between 1.25 mg and 4.16 mg.

These doses, transposed to man, correspond to values of 0.875 g to 2.912 g for a subject weighing 70 kilograms.

The animals were always sacrificed (killed) 20 to 30 minutes after ingestion.

B. Batches subjected to "aggressions"

The third batch was subjected to the action of aggressor agents: alcohol, chlorhydric acid, histamine, phenergan and histamine.

This third batch was subdivided into five groups:

The firsg group received alcohol according to the method described by Wegmann and Verne (1959). The alcohol employed titrates 56°. It is introduced by a soft eosophagus probe at doses between 0.2 and 1 ml per subject, i.e. 0.125 to 0.416 ml per 100 g of weight.

The total doses varied between 0.070 ml and 0.232 ml of alcohol at 100° per 100 g of weight.

This corresponds for a man weighing 70 kg to doses between 87.0 (? illegible figure) and 291.2 ml of alcohol at 56° of from 49 to 162.4 ml of alcohol at 100°.

The second group received chlorhydric acid: the method used is the same as the preceding, except that chlorhydric acid is introduced in the probe at pH 1.6. The volumes vary between 0.2 and 1 ml per subject, therefore from 0.125 ml to 0.416 ml per 100 g of animal weight.

In comparison with a man weighing 70 kg these doses vary between 87.5 and 291.2 ml.

The third group received histamine: the rats received either 1/2 mg to 2 mg of histamine bichlorhydrate solubilized in physiological serum 1 mg per ml or 10 mg of histamine in 2 ml of a solution at 30% polyvinylpyrrolidone.

The fourth group received phenergan before the histamine: ten minutes before the injection of histamine one injects subcutaneously 50 mg of phenergan in order to reinforce the secreting action of the histamine on the stomach (Halpern etal. illegible).

The fifth group protected by carrageen DB: These groups subjected to the illegible line .

Thus one group receives first of all alcohol and then carrageen DB in 1% solution; a second group receives first of all chlorhydric acid then carrageen DB at 1%; a third group to subcutaneous injection of histamine then carrageen DB at 1%; a fourth group first phenergan , then histamine and finally carrageen DB.

The animals were then sacrificed by dazing of the mind, then section of the carotid, either four hours after a single treatment or after four days of treatment done once a day. The organs removed were then frozen with liquid nitrogen (-193° C), then cut at the cryotome at -20° C. The illegible were then treated under accurate technical conditions perfected in our laboratory and published elsewhere.

The following reactions were made: the PAS reaction, the metachromase by the blue of toluidine, the glucose-6-phosphate dehydrogenase, the lacticodehydrogenase and the succinodehydrogenase.

Second Part

In this second part of the experiment we studied the action of carrageen on the psychic ulceration.

The method which we used differs from that related in the introduction, since preliminary research according to the method of Bonfils and Lambling caused a percentage of mortality very great associated with numerous ulcerations. Our purpose was to obtain sufficiently great lesions without risk of death.

The first group was subjected for 39 hours to fasting, that is to say deprived of food and water at will, in conjunction with a certain restriction in the freedom of movements. Each rat was closed in a small mouse cage made of plastic under conditions of visual and audio isolation. The animals were thus abandoned for 39 hours.

The control group, also deprived of freedom, received water and food in the form of oomplete biscuits. But it was difficult for them to reach the biscuits because of the restriction in movements. Under these conditions the quantity of food ingested is less than that taken by the animals living in group under so-called normal conditions.

To these two groups we administered either for 30 minutes or 2 hours before the sacrifice, a solution of 1% of carrageen DB at 1 ml per animal regardless of the animal's weight which varied between 160 and 210 grams.

After the sacrifice the tissues were treated as previously. We added, however, to the preceding reactions: total phosphorylase and malicodehydrogenase, the first belonging to the glycolytic channel and the second to the Krebs cycle.

REMARKS

Among the groups kept we very quickly eliminated those having received a strong dose of alcohol or chlorhydric acid. The alcoholic or chlorhydric shock, after a dose of 1 ml per subject, caused perturbations so serious of the morphology of the gastric mucosa that we were no longer able to keep these aspects for the histoenzymological study.

We remarked that under these very conditions the strongest doses of carrageen DB, i.e. 1 ml at a solution at 1%, were incapable of protecting the animals against the serious lesions. We therefore retained in all the rest of the experiment the weak doses of both the aggressive agent and the protective substance. The doses of the stress agent, related to man, were indeed rather great, those of the alcohol corresponded to the ingestion of a not abnormal quantity of alcohol, those of the chlorhydric acid, on the other hand, were clearly greater than the quantity secreted in 10 minutes in a normal man during digestion. Indeed, the recourse to this last experimental method was an attempt to simulate a hyperproduction of chlorhydric acid, to increase the quantity of pepsinogen, even though the hyperchlorhydria was not an indispensable factor for t he producing of ulcers.

Reactions made

They are the following:

A signaletic coloration at hemalun-eosine which yields only morphological data.

A coloration of blue of toluidine at pH 4.6 used as metachromatic histochemical reagent for the characterization of the acid mucopolysaccharides. A short discussion of the cellular "profiles" concerning the sites of production of the metachromatic substances is given in the discussion section.

A reaction to the green of methyl-pyronine or Brachet reaction which permits estimating the content of the cells in ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

A test of digestion by the corresponding enzymes, ribonuclease (RNA-ase) and deoxyribonuclease (DNA-ase) the role of which is to render the preceding reaction more specific. After RNA-ase since all of the elements captured the pyronine, therefore all of the elements containing RNA, both the ribosomes and the nucleoles, are negative, the RNA having been digested by the corresponding enzyme. After DNA-ase all of the elements having captivated the methyl green containing DNA , that is to say the chromatin of the nuclei, are digested.

The reaction to the periodic Schiff acid (or PAS) , also called McManus reaction, the positiveness of which indicates the presence of the sum glycogen, mucopolysaccharides (neutral) and glycoproteins.

This reaction indicates the presence of the mucoid and the mucins, whereas the reaction of the MPS concerns exclusively the acid mucopolysaccharides. This question will be treated further on since it is of primary importance.

The digestion by the amylases. We retained here the digestion by the amylase which exclusively degrades the glycogen of the normal tissues. However, we noted that the amylase is capable of also degrading other polysaccharides when their structure is modified or altered by the agents perturbing their molecular configuration.

The lacticodehydrogenase (LDH) is used to learn the behavior of the carrageen DH with respect to the principal metabolic path of anaerobic glycolysis.

The succinodehydrogenase (SDH) can be considered as the essential link of the Krebs cycle. We examined it in order to situate the activity of the product on the aerobic glycolysis.

The glucose-6-phosphate dehydrogenase (G6P-DH) constitutes a basic pivot of the entry towards the path of the pentoses or oxidative path the role of which is on the one hand to furnish the NADPH or nicotinamide adenine dinuclotide phosphate reduced, essential to the biosynthesis of the lipids, and, on the other hand, to lead to the formation of the ribose-5-phosphate and also to the deoxyribose leading to the deoxyribonucleic acid.

RESULTS

I. Appearance of the normal subjects.

A. Mucosa of the rumen

The PAS reaction is positive in the malpighian epithelium and only a very slight metachromasia is observed there. In contrast, the LDH is intense there at that level . The G6P-DH and the SDH have a moderate activity with respect to the former. We hardly noticed any basic changes in these structures under the experimental conditions.

c. Mucus

The mucus observed at the level of the gastric mucosa covers essentially the surface part of the epithelium with a coating forming a continuous film and lining the alveoles. This mucus is present at the level of the collar, in a relatively abundant proportion, less noticeable in the deep part of the isthmus, then reappears in the median part of the mouth of the glands and rarely reaches the bottom of the glands. The mucus is highly PAS positive. The digestibility by the amylases is rather marked. It presents a rather unmarked metachromasia at the level of the collar. It contains a moderate quantity of lactico-DH, but no trace of G6P-DH and SDH. This mucus fixes the radiomarked sulphate (Belanger,1954).

II. Normal animals treated with carrageen D.B. (Table I, Fig. 8 to 12).

No clear change is noted at the level of the rumen. However, at the gastric mucosa one notes a considerable drop in the PAS activity. All of the cell elements give the impression of not reacting to the periodic Schiff acid. The mucus at the surface of the mouth of the stomach is not very abundant. On the other hand it is very abundant at the foveoles, stopping sharply at the collar. The density of the mucus appears to be much greater than in normal mucus. However, one notes,at the same time as an increase in the basophilia of the cells of the foveoles, an increase in the metachromasia at the level of the collars and the upper part of the isthmus. The reaction of the G6P-DH is depressed as a whole to various degrees. The lactico-DH and the SDH apparently do not present any variations at all. One notes a great increase in the basophilia and in the rate in RNA at all levels.

III. Aggression by chlorhydric acid or alcohol (Table II, Fig. 1 to 4).

One notes a rather distinct decrease in the mucus at the level of the foveoles with respect to normal, but the depression of the PAS activity of all of the cells is less important (not as great) after D.B.One notes no changes in the metachromasia, but an appearance of G6P-DH activity at the level of the foveoles (II,2).

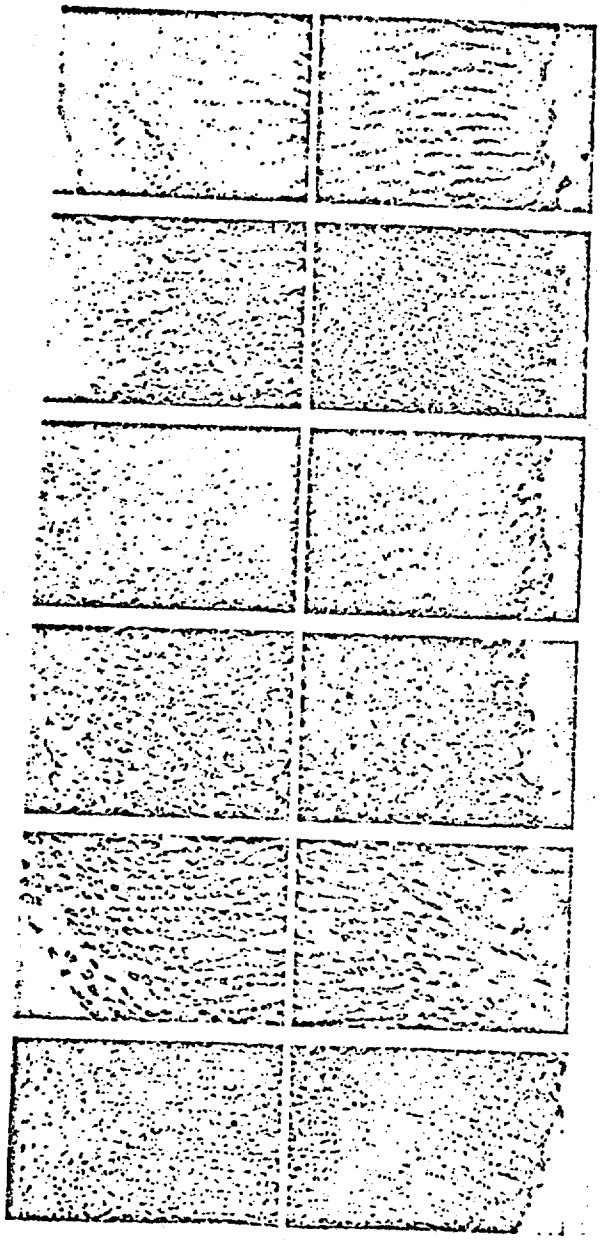
This action is even increased when the chlorhydric or alcohol treatment is extended over 4 days , to a moment when the mucus tends to disappear from the epithelial surface (II,1). In contrast, the LDH (II,3) decreases considerably to the point of disappearing, at the same time that the upper part of the foveoles assumes a rather strong charge of SDH (II,4), effects which were similar after alcohol treatment.

IV. Protection by carageen D. B. after HCl or alcohol (Table II, Fig. 5 to 8).

The protection with carrageen DB both during a short period of time and during a longer period of time (4 days) brings on the appearance of a very large mucoid layer (II,5), whereas the various cells resume an appearance and a reactivity to PAS close to normal, digested partially by the amylases. The G6P-DH (II,6) is clearly depressed with respect to the stress agent, without however inhibiting the activity, as observed in normal tissues. The LDH (II,7) returns to normal and the SDH ((II,8) also. As concerns the various types of cells during these reactions, above all parietal and principal, they have a tendency to resume an appearance close to normal, except for the LDH which is a bit more intense. The rate in RNA and DNA therefore appears to be normal.

TABLE I

NORMAL CONTROL GROUP



CONTROL GROUP + DB

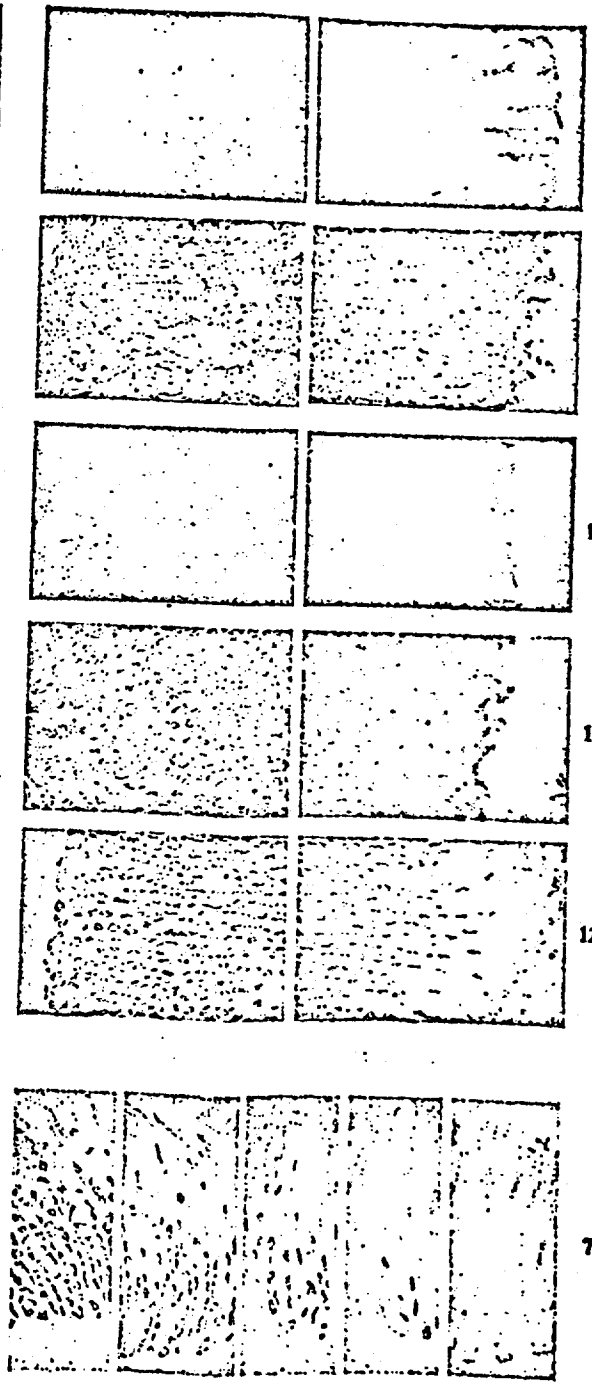


Table 1

Remark: All of the cuts were obtained fresh with cryotome: thickness of 5 μ. In each column we represented : to the right the upper third of the mucosa and to the left the lower third. The middle one-third not shown is very close to the areas which are contiguous on the 2 neighboring photos.

A) The gastric fundic mucosa represented corresponds for each series. Only the upper and lower thirds of the fundic mucosa are shown.

1. PAS" Abundant mucus at the level of the epithelium and foveoles, discreet in the collar, the isthmus and close to the upper part of the indicated glands.
2. Toluidine blue (BT): Diffuse metachromasia in the foveoles but not in the isthmus, on the other hand 1/3rd of the fundic glands has a diffuse metachromasia, which is to be superposed to the PAS reaction at this level.
3. Glucose-6-phosphate dehydrogenase (G6P-DH): the epithelium (surface) is moderately active, as well as the cells bordering in the medium part of the glands and at the bottom.
4. Lacticodehydrogenase (LDH): Medium reaction in some areas of the epithelium and a bit more clear in the bordering and principal cells of the lower third of the glands.
5. Succinodehydrogenase (SDH): Activity zero in the foveoles and the collar; very discreet in the principal cells and very strong in the bordering cells.
6. Hemalun-eosine: Notice the various regions and from right to left: epithelium foveoles, collar, isthmus predominantly acidophilic (bottom) and glands.
7. Succinodehydrogenase of the bordering cells in the five sectors of the gastric mucosa from fundus (to the left) to the pylorus (to the right): The number of bordering cells, abounding in the fundus, diminishes in the direction of the pylorus where they are absent.

B) Effects of the carrageen DB: Fig. 8 to 12 (magnification x 81)

8. PAS: One notes a greater concentration of the mucus in the foveoles: the mucous film at the surface of the epithelium is thin. The cellular and mucous reactivity of the fundic stomach has diminished very clearly.
9. B. T.: Basophilia and metachromasia of the foveoles have increased. The isthmus is not very active. The glands have the same metachromasic and basophilic characteristics as the foveoles.
10. G6P-DH: The sedation of this enzyme remains very clear at all levels.
11. LDH: Increase in the activity in the surface epithelium and the foveoles. Rather diminution in the gastric glands.
12. SDH: Few considerable changes with respect to the normal subject. Note, however, that the principal cells (adelomorphous) are no longer active.

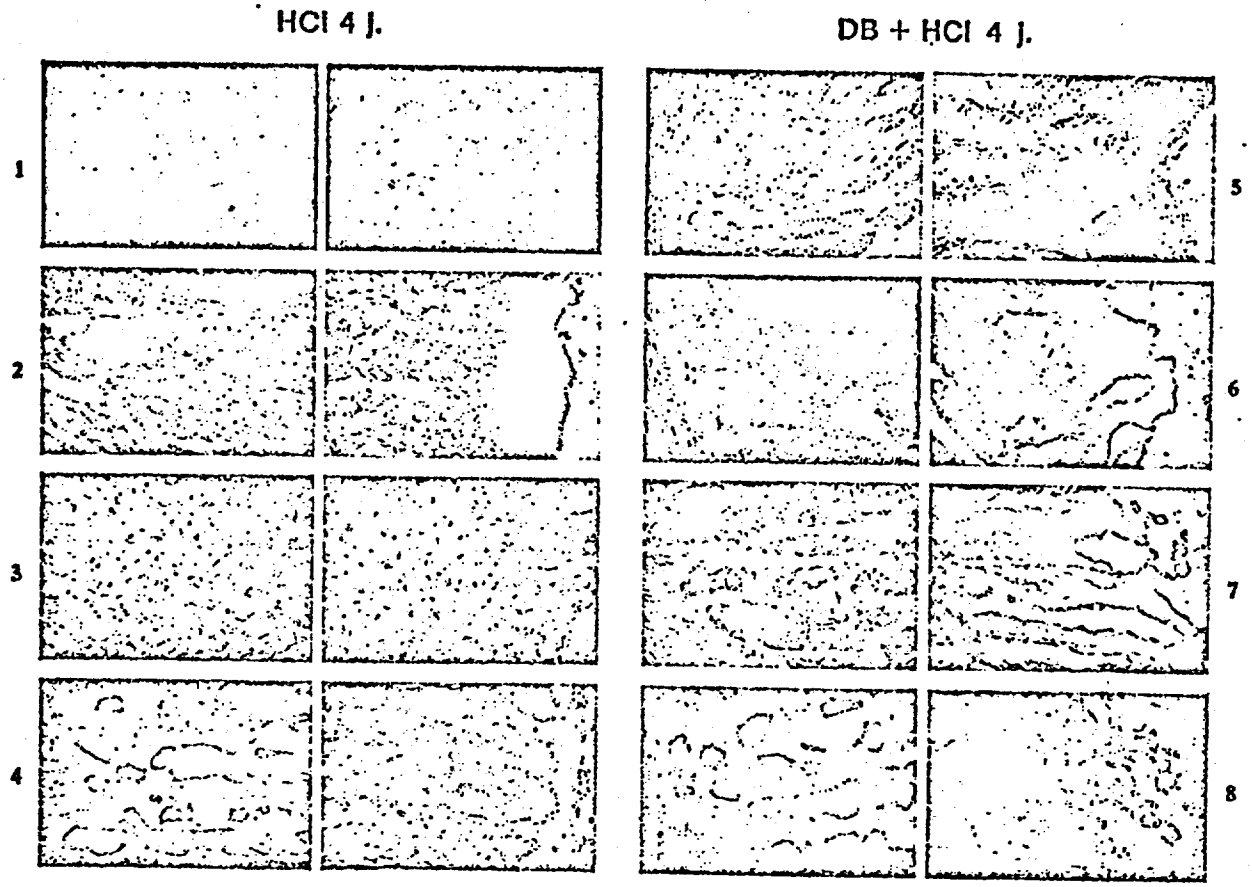


TABLE II

A) Chlorhydric acid for 4 days: Fig. 1 to 4 (magnification x 204)
1. PAS: Very distinct sedation in the mucus and in the cell structures.
2. G6P-DH: Distinct increase in the mucus, the foveoles and in the parenchyma.
3. LDH: Very distinct sedation in all of the cells and at all of the levels.
4. SDH: Increase in the activity in the lining epithelium. The bordering cells of the collar are emptied of their secreta.

B) Protection by the carrageen D.B.+ HCl (4 days): Fig. 5 to 8 (magnification x 240)
5. PAS: Appearance of the mucosa and mucus about normal.
6. G6P-DH: Activity still distinct in the surface epithelium and the foveoles, but less than Figure 2. Notice that the principal cells are widely active.
7. LDH: The activity has once again become approximately normal at the surface. In the glands the principal cells have the same positivity as after carrageen DB alone.
8. SDH: Return to normal activity at all levels.

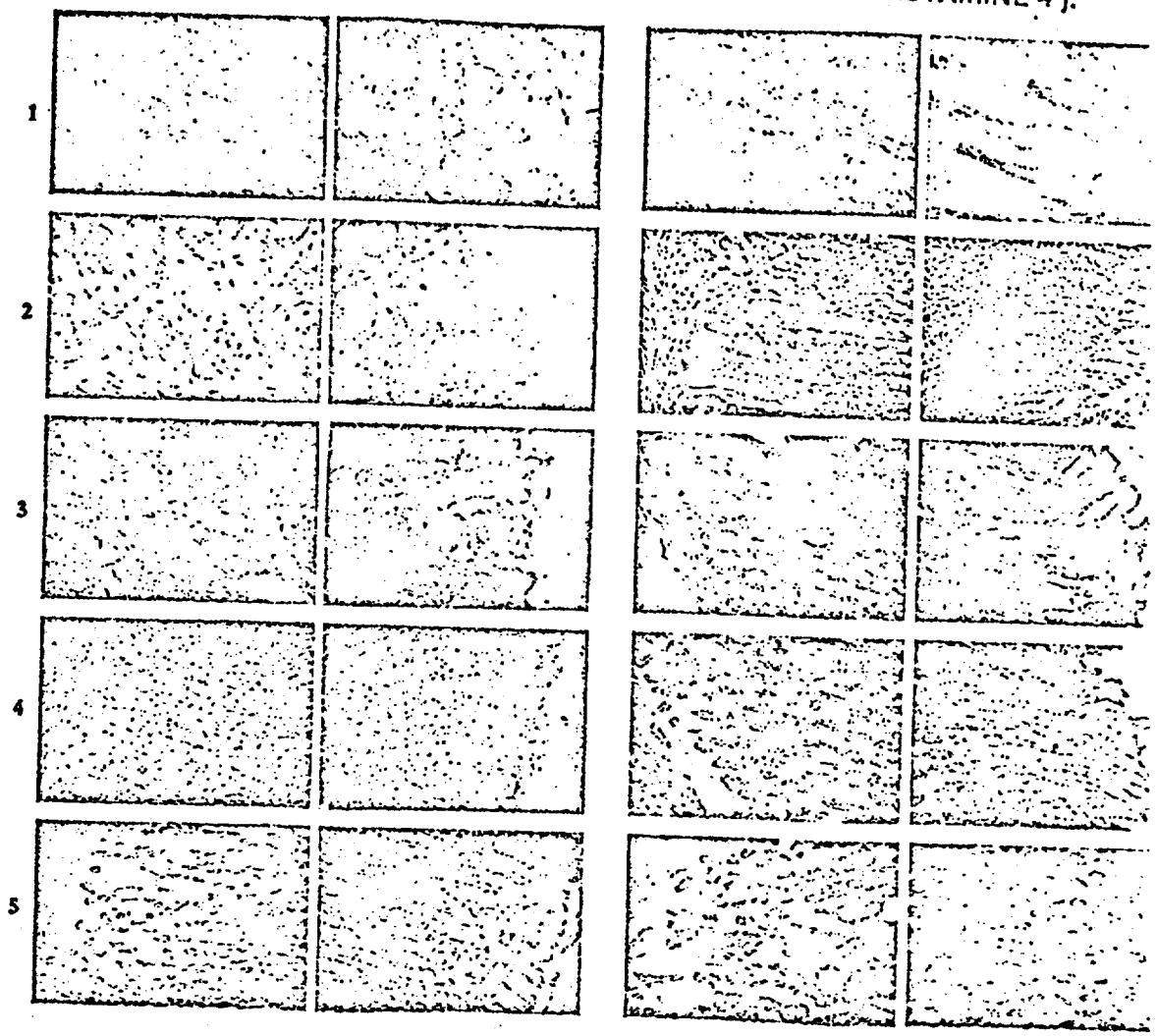


TABLE III

Fig. 1,2,3 x204; the other figures x 81

A. HISTAMINE FOR 4 DAYS (Fig. 1 to 5).

1. PAS: Strong inhibition of the mucus.
2. B.T.: no metachromasia; bordering cells very clear, hypertrophic.
3. G6P-DH: Increase in surface activity. As for the rest, rather diffuse.
4. LDH: Increase at surface, inhibition moreover towards the bottom.
5. SDH: We observe the absence of SDH in numerous bordering cells, particularly at the bottom of the glands.

B. PROTECTION BY CARRAGEEN AFTER INJECTION OF HISTAMINE FOR 4 DAYS (Fig. 6 to 10).

6. PAS: The surface and the foveoles are rich in mucus, but the bottom of the glands remains relatively inactive.
7. B.T. (x81): Distinct metachromasia at the collar and isthmus. The bottom also presents this, but in a more diffuse manner.
8. G6P-DH: A certain modification with respect to the stress is obtained. The cells are still positive at the surface.
9. LDH: Distinct tendency towards improvement and return to normal.
10. SDH: Appearance normal as in the control group animals not treated.

V. Aggression by histamine or histamine-phenergan (Table III, Fig. 1 to 5).

Treatment by massive and sole injection of histamine or phenergan, or the two together, causes a considerable increase in the mucous secretion wigh a reduction in the reactivity of the parenchymatose cells (III,1). On the other hand a treatment extended over a period of 4 days of histamine or phenergan-histamine causes an inhibition of the mucus and of the mucous and gastric glandular cells. The basophilia diminishes (III,2) and the mucous assumes a diffuse appearance in its intensity both for the G6P-Dll (III,3) and the LDH (III,5) and, to a lesser extent, for the SDH (III,5).

VI. Protection by carrageen D. B. after histamine (Table III, Fig. 6 to 10).

The protection under all of these conditions by the DB 15-10 causes an increase in the mucous secretion (III,6), a reappearance of the metachromasia (III,7) in the principal cells, but not in the cells of the foveoles, a clearing up in the mucous and glandular cells both for the SDH (III,10) and the LDH (III,9) or the G6P-DH (III,8). However, although the LDH and the SDH are approximately normal, the G6P-DH remains still active although very moderately.

VII. Effects of the psychic stress (Tables IV to VIII, Fig. 1 to 6).

In addition to the preceding reactions we have also studied the malicodehydrogenase, phosphorylase and compared the animals placed on fasting with animals which were fed.

For the LDH (Table IV, Fig. 1 to 6) the activity at the mucus and collars is zero. The number of bordering cells is greater in the fed subject than in the fasting subject. If the two subjects are protected by the DB 15-10 the differences are not great, while the G6P-DH (Table V, Fig. 1 to 6) is a bit more intense in the collar and chorion and rather in the foveoles in the fed subject.

As concerns the SDH (Table VI, Fig. 1 to 6) the variations observed on fasting are rather small and almost non-existent at the epithelium. However, in the fed subject the activity, which is very average at the beginning, becomes relatively intense 2 hours after the administering of the carrageen DB.

The MDH (Table VII) reveals a rather considerable increase 30 minutes after the ingestion of the extract, both under fasting and in the fed subject. The epithelium in the fasting subject then remains active, whereas in the fed subject it drops clearly below a normal value to become almost negative.

The phosphorylase (Table VIII) does not reveal any characteristic activity, except for 2 hours after ingestion of the extract, both in the fasting subject and in the fed subject.

GENERAL DISCUSSION

From these experiments as a whole we note above all that the stress (external: chlorhydric acid - alcohol),by parenteral means (phenergan and histamine) and psychic (fasting) cause considerable changes in the reactions the modulation of which depends largely on the nature and path of introduction of the stimulus changing the gastric metabolism.

The first point which should draw our attention is that of the polyvalent action of the extract of the Chondrus crispus on the gastric secretion both on the various types of mucous cells and on the parietal and peptic cells.

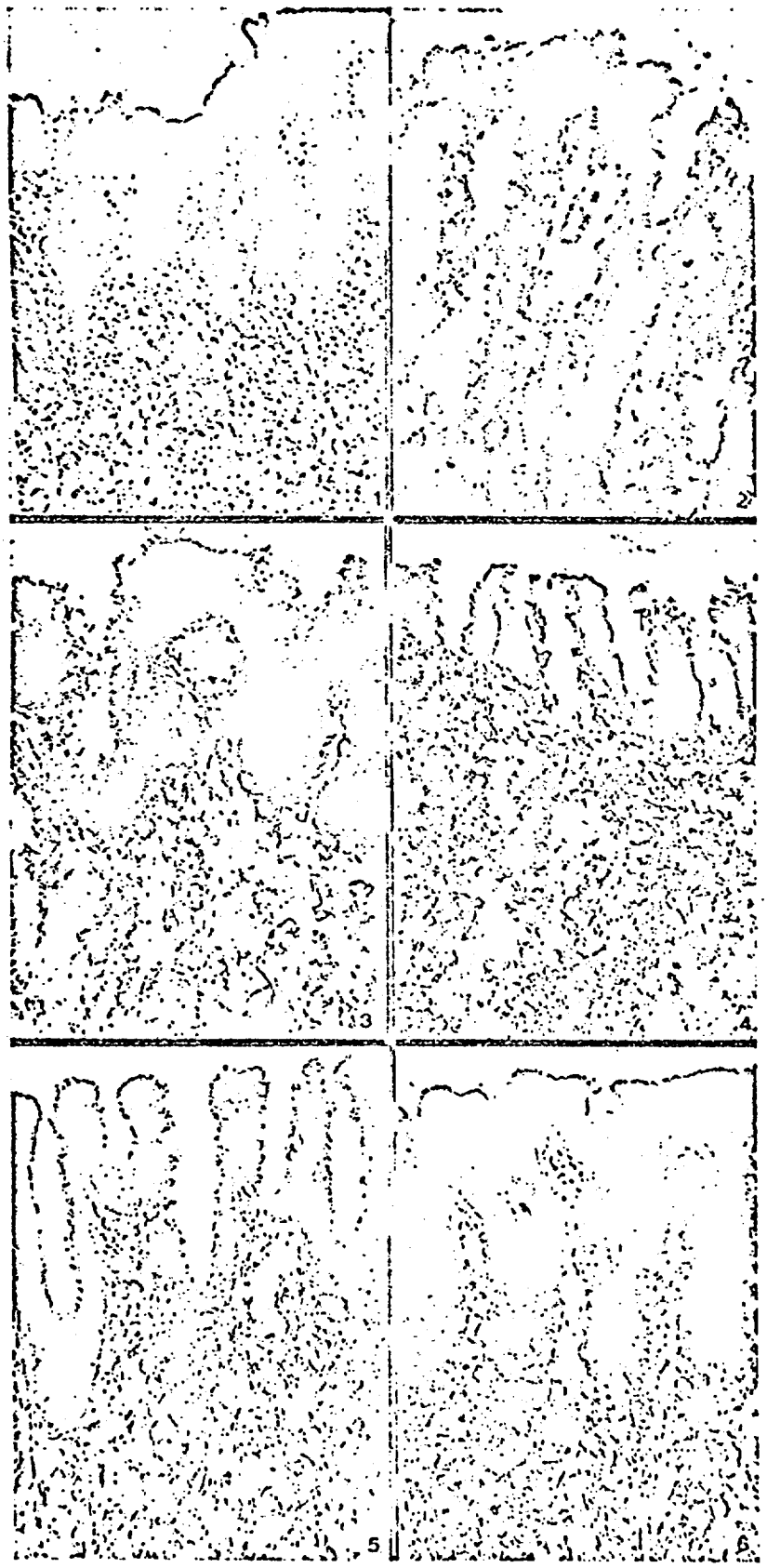
LACTICO DH

Table IV(LDH)

FASTING FED

T

2 h



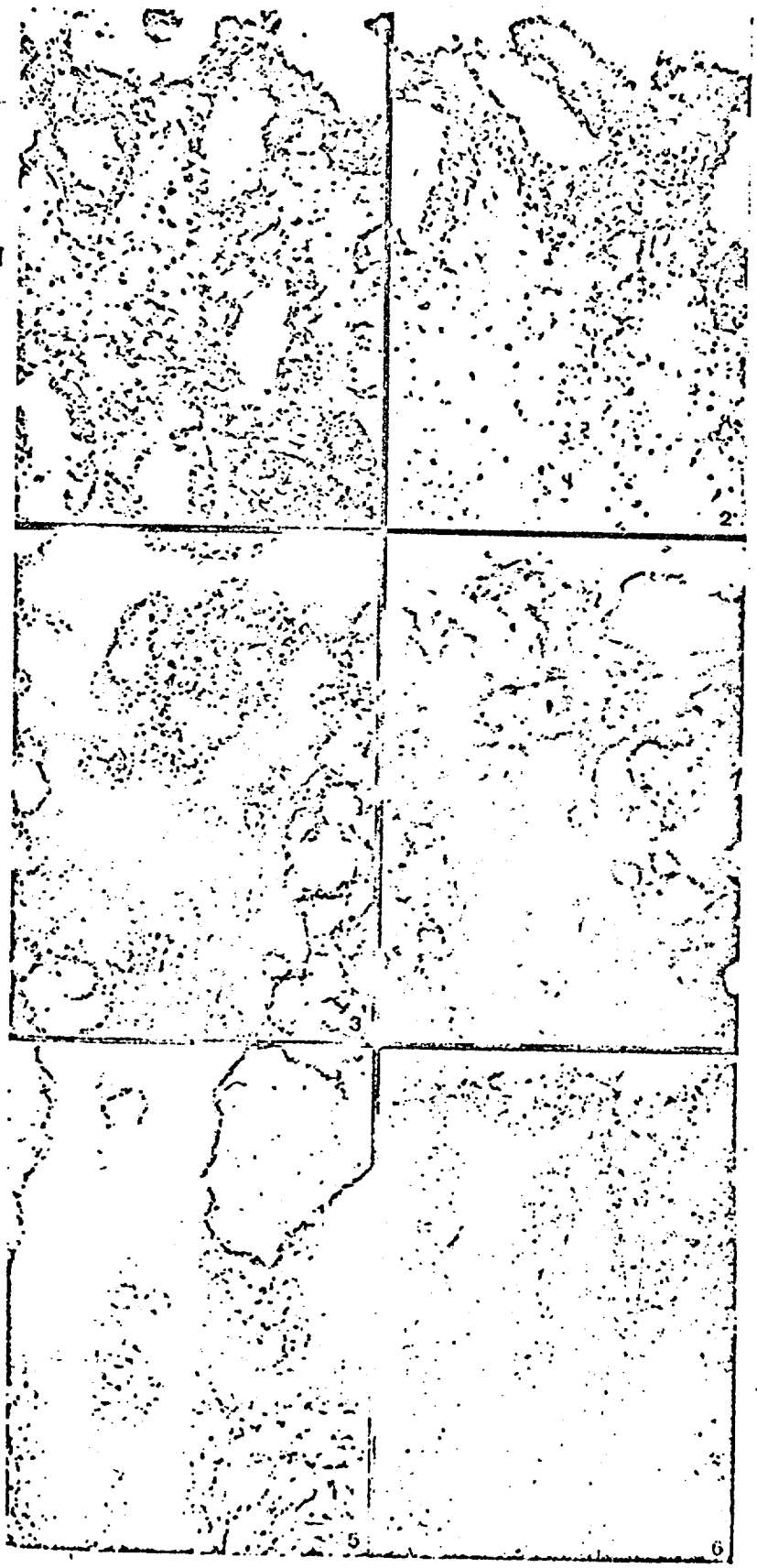
A. Fasting:
1. Average activity of the surface epithelium and foveoles.
3. The foveoles are dilated and the activity increases
5. The foveoles are dilated and the epithelium is positive over the entire surface.

B. Fed:
2. The foveoles are contracted and their epithelium moderately positive.
4. The foveoles are not very deep and moderately positive.
6. Very distinct increase of the surface epithelium and the foveoles up to the collar.

G.6.P DH

TABLE V (G6P-DH)

FASTING FED



A) Fasting:

1. Very weak reaction in the surfac epithelium. Some foveoles are positive at the surface but not deeper.
3. Considerable increase in the activity of the surface epitheli and of the foveoles which extend to the collar.
5. Very strong action in the mucus, in the lining epithelium and a bit weaker in the collar.

B) Fed:

2. Average activity in the surface epithelium
4. Non-reinforced activity at the surface, but at the level of the foveoles where we observe a very positive mass, probably mucus.
6. Activity absent in the surface epithelium and present, but only slightly, in the foveoles.

This action is exerted despite the fact that there is no absolutely constant profile of secretive or enzymatic activity of the various cell varieties. Thus for example if we compare the so-called normal appearannes of the G6P-DH of the control animals used for the external aggression experiments (chlorhydric acid or alcohol), internal aggression by parenteral means (histamine or histamine phenergan) or by psychic aggression (fasting), we note some differences both as regards intensity and location. This does not prevent us from obtaining an idea as to the so-called average activity of these enzymes or macromolecules (the mucopolysaccharides for example).

Furthermore we noticed that the various aggressions do not exactly produce the same type of cellular response. Thus, chlorhydric acid in a single dose in a subject during period of digestion rest causes a brutal mucous hypersecretion, associated with enzyme changes which differ from those observed when this acid is administered for four days, which inhibits this secretion. This remark is valid for all of the other aggressions, single or prolonged, the responses of which are caused, on could say, specifically.

In all of the cases for the purpose of controling the effect of the carrageen DB we retained the effects of prolonged aggression which are precisely the most difficult to reverse. And what did we observe? Most often we observed a regularization of the processes either in the sense of an inhibition of an exaggerately high effect or in the sense of an activation when the system has been blocked by the stress. We even saw, after 4 days of chlorhydric acid, that this extract can at the same time stimulate a system which has been inhibited (the LDH) and partially block another system (the G6P-DH) which has been very greatly excited.

Most of the time, therefore, the carrageen DB seems to exert a "buffer" effect which is very complex and which does not involve only the mucous secretion. We were thus surprised to see that this product, the reaction of the PAS serving to characterize the polysaccharides and the neutral mucopolysaccharides, renders, as a whole, the gastric mucosa non-reactive to the PAS reaction, but causes the depositing of a very thick film of mucus in the foveoles above all. At the same time the G6P-DH is inhibited but not the LDH and the SDH. Under these experimental conditions by the carrageen DB the reactivity to the PAS after aggression is increased both in the gastric mucosa and in the mucus, but that of the G6P-DH is almost always depressed.

We can therefore deduct that the effect of this extract of Chondrus crispus is much more complex than that of an antipeptic or antacid substance. It activates without any doubt the secretion of the mucus, but an increase in the secretion of the mucus is not necessarily a beneficial result. The proof of this is the brutal increase in the quantity of mucous secretion after a single dose of chlorhydric acid, histamine or histamine-phenergan. It is therefore of no use to wish to solely increase this mucous secretion in quantity. It is necessary to act on all of the cellular activities. This is what we observed during this experiment, although we did not involve in an evident manner the so-called pyloric secretions. Indeed, the absence of enzymatic reactions characteristic of this last level did not seem to us to be a great handicap to evaluate the effects of this substance at the gastric mucosa as a whole.

The fundus and pylorus form a coherent whole, despite the diversity of the cell proportions.

The problem is then placed, above all, to learn the nature of the mucous secretion obtained after administration of the extract. In the normal subject it does not form any mucus in a quantity exceeding the normal. This fact is only observed after the aggression. It is therefore necessary to admit that the carrageen DB has no "mucigenic" (mucous-producing) power in the sense usually meant. After alcohol or acid, for example, it also does not seem to wish to unite with the mucus of the normal gastric surface, since the protective layer formed hardly seems greater than normal, except in the foveoles. Is this effect indirect by action on the cells of the collar and on the cells of the lining epithelium? Then why is the rest of the mucous seen by means of the PAS so little active?

A start of the answer seems to be provided from examination of tables IV to VIII where we note that the mucous film caused on the surface of the gastric mucosa varies according to the duration of administration of the carrageen DB. By way of example, the G6P-DH in the fed subject sacrificed 30 minutes after absorption of the product is more active than after 2 hours of effect (V,6). But it should not be forgotten that these animals experienced a certain stress closed in a cage where they could hardly budge. We see therefore that the conditions differ from that of subjects which have not been deprived of their freedom. This perhaps explains the differences observed. In all of the cases the normal rat, without constraint, reacts, throughout the level of the mucosa, by a very considerable drop in the G6P-DH activity (Plate I, 10). It is therefore necessary to admit that the psychic factor plays a determining role, resulting in very visible biological and enzymological phenomena.

The complexity of the responses to the psychic stress, however, seems to indicate the participation of a greater number of biochemical elements and factors than an external stress (simple alcohol,HCl) or an internal stress (histamine, phenergan).

It seems, however, that the protective agent use is not a simple coating agent of the gastric mucosa but that it intervenes by inherent metabolic effects involving all of the types of secretion. In this sense the fundic gastric mucosa of the rat is not very different from that of man based on the histochemical and autohistoradiographic characteristics established by the work done by Cornet et al. (1960 a and b). Is this an antipeptic effect, however, as assumed by Bonfils et al. (1966)? Those researchers studied the effects of a carrageen in vitro,whereas we examined the activity of the carrageen DB in vivo. In this case, probably, both the chemical state of the substance and the physiological state of the subject must play a role. But how? According to Anderson (1961 and 1962), the peptic inhibiting effect would be parallel to the rate of sulphate contained in the polymer and Ravin et al. (1962) show that there is proportionality between inhibition and sulphate content if the content is greater than 15% of the product.

Furthermore, the carrageen DB is capable of being combined with beta-lipoproteins (Gros and Laudat, 1963), which is equivalent to a clarifying factor and thus increase the capillary resistance in order to act as an anti-coagulant agent (Cook et al. 1963).

FASTING FED TableVI

A) Fasting:

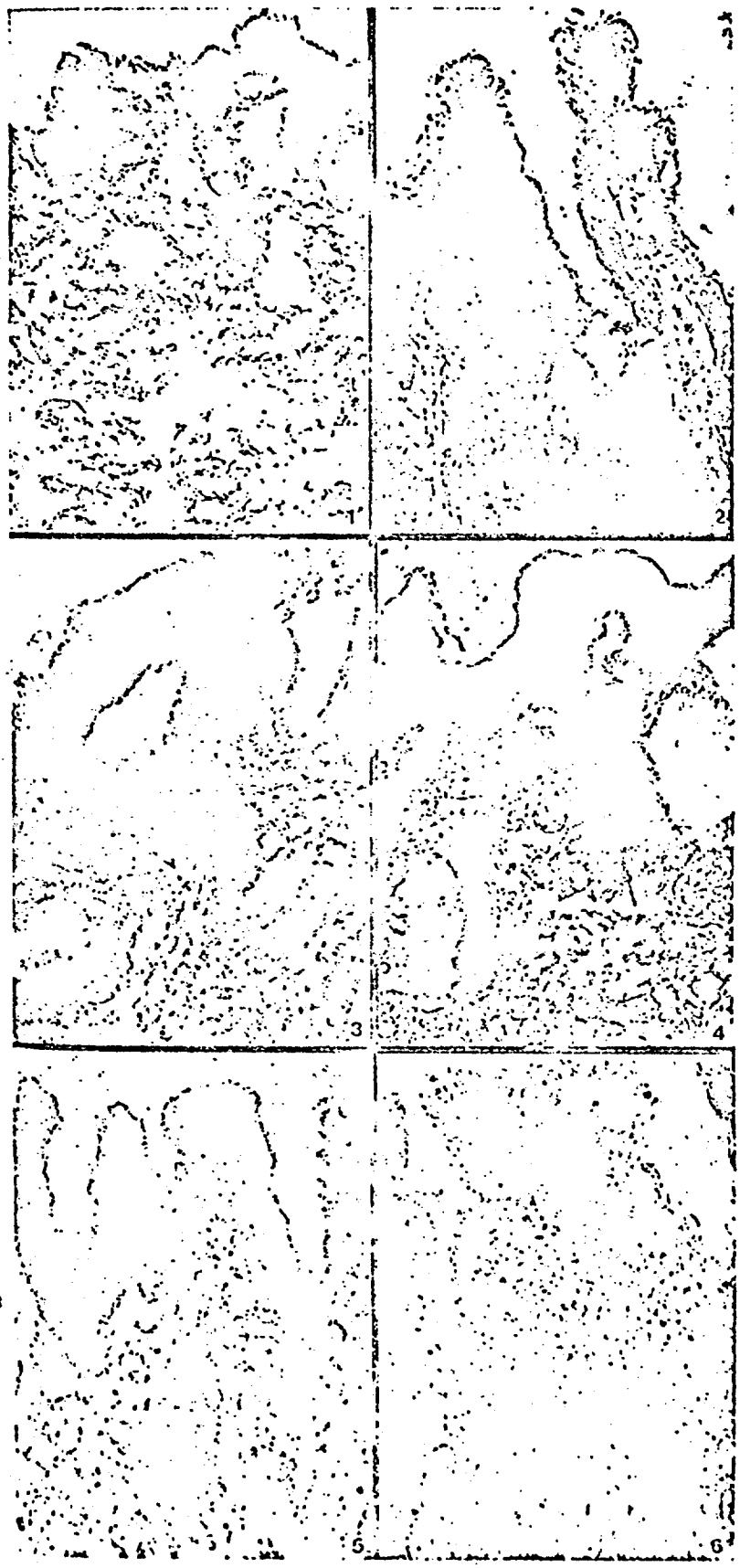
1. The zero activity in the epithelium and in the foveoles, and average in the bordering cells of the collar
3. Distinct activity of the bordering cells near the collar
5. Absence of activity of these same cells, trace of reactivity at the level of the surface epithelium.

B) Fed:

2. Average activity in the bordering cells and more numerous at the collar
4. Intense activity at the level of the bottom of the foveoles and at the level of the collar, part of the bordering cells.
6. Increased activity in the bordering cells with respect to the control animals.

SUCCINO DH TABLE VII (SDH)

FASTING FED



A). Fasting:

1. Distinct activity at the epithelium and less intense at the foveoles.
3. Increase in the activity in the epithelium and the foveoles which are dilated.
5. Distinct activity in the epithelium and somewhat less intense at the bottom of the foveoles.

B). Fed:

2. Intense activity in the epithelium and in the chorion.
4. Increase in the activity at the epithelium (surface) in the chorion and in some portions of the foveoles.
6. Very distinct decrease in the epithelium in the foveoles and in the chorion.

Does this product possess an antacid effect as many simple bodies? This is difficult to prove because its amphoteric action gives it moreso a buffer power. Furthermore, the electrically negative charges of the mucopolysaccharides which it includes can neutralize the basic proteins (Fischer and Astrup, 1965 - Jacques, 1943) just as the proteins associated with the carrageen DB can combine with the tissular mucopolysaccharides. This double-flow action can perhaps explain to a large extent the close relationships of this body with the proteins at the level of the cell.

For each cell protein, however, the relationships will be of a different nature. We know for example that in pepsin there are 4 different types of proteins at pH isolectric between 1 and 3 (Bonfils et al., 1966) and we know the large number of anionic residues which exist in excess in the pepsin with regard to the cation residues, therefore aspartic acid and glutamic acid with respect to arginine, histidine and lysine (Lillie, 1958).

If we allow this carrageen-protein relationship or bond we must then contemplate multiple bonds with cellular proteins and if we think that potentially in each cell there are about 700 enzymes then this substance would probably link up with all of the proteins at this level. As far as we are concerned, it seems that it is these bonds which cause all of the effects observed and, in particular, the curious and very great affinity for the glucose-6-phosphate dehydrogenase, whether or not this protein-enzyme is intact or not. What will probably regulate the "protective effect" is the pH of the medium, the carrageen then retaining in a strongly acid medium its amphoteric nature. The same should hold true for all of the other enzymes and metabolites, the compound formed always remaining easily dissociable.

We can no longer speak therefore of an antipeptic effect. We should rather speak of a multi-antienzymatic action, the pepsin being only one of all of the other enzymes considered on which the component acts. Thus, for example, this extract of Chondrus crispus could or could not be an inhibitor of the intrinsic factor of Castle which we know occurs in the principal cells in the rat and in the parietal cells in man (Hoedemaker et al., 1964) or the production localized at the level of the lining epithelium of the gastric mucosa (Fujie et al., 1957 and 1958) or else vis a vis the parietal anticell antibodies secreted probably by the stomach (Fischer et al., 1965). If we know that the region of mitosis, normally localized in the area of the collar, extends at the time of the gastric erosions until it replaces the epithelium (surface) and in the ulcers on all of the ulceration (Kaku, 1966) , we can ask ourselves whether certain actions of the carrageen DB would not consist in stimulating these mitoses in the case of aggression (erosion by alchol, HCl, histamine, etc.), by its liaison with either the nucleic acids or with theirs synthesis enzymes. This element should be confirmed. The fact is that if the normal coating of the stomach is lacking and if a permanent reflux of the duodenum damages this surface we then note a progressive atrophy of this mucosa (Belowski et al., 1961). But the ulcer or the cancer increase in the stomach the rate of mitotic renewal as during the course of the hypophysectomy (Peremans, 1957). The mucus is therefore a barrier of metabolic exchanges in both senses, but its absence breaks the equilibrium, hence atrophy. We were above all astonished by the fact that it was not sufficient to maintain the mucous film, it is absolutely necessary to respect the metabolic exchanges.

TABLE VIII

(Total phosphorylase)

PHOSPHORYLASE

FASTING FED

Table VIII

A) Fasting:

1. Zero activity in general.
3. General increase in the coll exclusively.
5. Increase in the collar and t upper part of the isthmus (at the bottom of the photograph).

B) Fed:

2. Very slight (discreet) activity at the surface epithelium and the collar.
4. Increase in activity in the bordering cells of the collar and isthmus.
6. Widespread (diffuse) increase in the epithelium of the chorion and the isthmus.

The idea that there is a liaison with the cell proteins postulates a penetration of the product into the cells, including the parietal cells. Indedd this does not seem to be impossible or improbable to us, but then it would be necessary to use autohistoradiographic methods. There could be another mechanism in the case of the bordering cells: the acid secreted would be absorbed by the carrageen DB, in view of the great role of the molar concentration of a "fixating agent" on the parietal cells (Vial et al., 1966), thus preventing the effect of the histamine, alone or potentialized on the phenergan, on the stomach, which was discovered by Halpern et al. (1946) and Brun (1952) Birnbaum et al. (1958) and Ishihara et al. (1960) had shown to be a direct effect and not a neural or neuro-humoral effect. Legeron et al. (1965) showed a biphasic effect of the histamine on the gastric SDH, stimulating both the basal part and the apical part of the mucosa, which seems to reveal a difference composition of these 2 regions connected or not to a particular receptivity of these sectors. The inhibition of the acid secretion by the extract of Chondrus crispus would not be different from that obtained by Stoffels et al., (1966) after use of inhibitor (PCMB) of the sulphydril groups, but concerning which we do not know if they block the system producing the energy or a particular enzyme invervening in the secretion of hydrogen ions. In contrast the EDTA (or ethylene diamine tetracetate acid) does not block the SDH of the cells producing the mucus (Rosan et al., 1960). But there too, according to the case, the carrageen DB acts in the cases of acid hypersecretion (artificial) as an inhibitor and in the acid hyposecretion (achlorhydria) as a parasympathomimetic activator (Villareal et al., 1955).

Finally, carrageen would not act as the habitual modifiers of the rate of mucus of the gastric surface, among which Flavian (1957) placed in first place the subnitrate of bismuth. It would act, rather, first of all on the surface cells, then on the cells of the collar which, in addition to their power to secrete mucopolysaccharides, posses a distinct lipasic activity (Abe et al., 1964) and phosphatasic acid (Ragins et al., 1964). It is only later, according to us, that it would act on the other cell varieties: but for that a certain duration of presence of the substance is necessary , as indicated by the more favorable effects if the protector is administered for several days, unless the subject has experienced a psychic stress , in which case the useful response is obtained earlier without forgetting that the conditions of fasting and feeding are not negligible.

Furthermore, the difference between the two states (fasting and feeding) was well pointed out by Helander (1964) by electron microscopy, where the principal cells experience above all an increase in the size of the grains during the renutrition and the parietal cells, above all their microwillosities, become first of all smaller in size and then expanding.

The importance of the carrageen DB would reside, according to us, in limiting or preventing the gastric loss of proteins during severe damages of the mucosa and, very exactly, the gastric epithelium, in humoral lesions such as the acute and glcerous gastrites in the inflammatory diseases (Jarnum, 1964) and the hypertrophic chronic gastritis which has nothing in common with the chronic gastric hypertrophy.

Preto Parvis et al. (1962) showed by electrophoresis important differences in the composition of the various secretions of mucus in the gastric mucosa, although the same type of mucus can conceal numerous varieties of substances the exact role of which escapes us. Mucus is a compound, not a definite chemical body, and it is this compound which the carrageen pretends to replace while awaiting a normal cellular secretion. This is not only a gastric dressing but also a substitute for the gastric secretion. It is probably from this that it gets its so delicately modulated effect.