Protective Effect of Seaweed Extracts for Chicken Embryos Infected with Influenza B or Mumps Virus. (24429)

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In screening tests for antiviral activity we often observed that insoluble compounds prepared as a suspension in 0.25% agar exhibited erratic, minimal protection for chicken embryos infected with influenza B virus. We suspected the presence of an active material in the agar, the more so, because different batches gave different results. This prompted the present study of the polysaccharides obtained from marine algae, particularly *Gelidium cartilagenium*, the common source of domestic agar, and carrageenan, a galactan derived from *Chondrus crispus*. Polysaccharides from various sources exhibit certain antiviral properties. Bacterial polysaccharides have shown marked antiphage activity (1,2) and a yeast polysaccharide inhibited the multiplication of tobacco mosaic virus(3). The modifying effect of bacterial polysaccharides on infections caused by animal viruses was first observed by Horsfall and McCarty(4) and later by Ginsberg, *et al.*(5). They demonstrated the marked antiviral specificity of these substances. Similarly apple pectin had specific effects against influenza A virus in fertile eggs(6).

Materials and methods. Seaweed extracts. Gelidium cartilagenium: Agar obtained from this and other species of seaweed is mainly a linear polysaccharide of D-galactose units linked 1→3, with a small number of ethereal sulfate groupings(7). A typical extract was prepared as follows: 56 g of desiccated seaweed* was allowed to swell in 4 liters of distilled water, homogenized for 1 hour in Waring blendor, and shaken for at least 24 hours in a stoppered bottle. The insoluble materials were separated by centrifugation and filtration through paper, after which they were held for future extraction. After the filtrate had been concentrated *in vacuo* (in a falling film evaporator at 40°C) to 1 liter, the polysaccharide was precipitated by addition of 2.5 l of acetone. The collected precipitate was redissolved by warming in 125 ml of distilled water and the solution lyophilized to yield 3.75 g of a colorless solid. The insoluble seaweed material, processed a second and third time, yielded 1.86 g and 0.75 g of lyophilized solid, respectively. *Chondrus crispus*: Aqueous extracts of this alga yield the commercial product, carrageenin, which when purified is called carrageenan. It is a polysaccharide consisting principally of D-galactose units with some L-galactose and a ketose present. The amount of ester sulfate is about 28% greater in carrageenan than in agar. Carrageenin† was dissolved in water and acetone precipitated as described under *Gelidium* above. The lyophilized samples prepared from both sources gave typical test reactions for polysaccharides and were low in ash (2 to 5%). The component associated with antiviral activity was non-dialyzable. *Solutions.* Antibiotic broth was prepared by addition of 100 units of penicillin and 100 mcg of dihydrostreptomycin/ml of tryptose phosphate broth. Glucosol solution was prepared as described by Fulton and Armitage(8). *Viral strains.* The WS and PR8 strains of Influenza type A, Lee strain of Influenza type B, Lasota strain of Newcastle disease virus (NDV) and Enders strain of mumps virus were employed. All except mumps virus cause lethal infections in 10-day-old embryonated chicken eggs. These viral strains were stored as infected allantoic fluid in sealed ampules a –70°C. *Titrations of infectivity.* Infected allantoic fluids were diluted in 10-fold steps in antibiotic broth. Groups of four 10-day-old embryonated eggs were inoculated intra-allantoically with 0.2 ml of each dilution and incubated at 36°C for 2 days or, in the case of mumps, for 6 days. The presence of viral hemagglutinins in the

* Supplied through courtesy of American Agar and Chemical Co., San Diego, Calif.

† Seakem No. 6, obtained through courtesy of Seaplant Chemical Corp., New Bedford, Mass.

TABLE I. Effect of Algal Polysaccharides on Viral Infections in Chicken Embryos (Treatment and Infection by the Allantoic Route).

Material inj. 1 hr after infection	Mumps	Influenza							
		Lee		PR8		WS		NDV	
	I/T*	S/T†	AST‡	S/T	AST	S/T	AST	S/T	AST
1.25 mg carrageenan extract	2/10	9/10	>10.0	0/10	5.6	0/10	4.4	1/10	6.7
1.25 mg *Gelidium* extract	3/10	7/10	9.6	0/10	5.8	0/10	4.6	0/10	6.9
.5 ml antibiotic broth	10/10	0/10	4.2	0/10	5.2	0/10	4.7	0/10	6.7

* I/T = No. of infected/total; 100 EID_{50}.
† No. of survivors/total; 100 LD_{50}.
‡ Avg survival time in days.

allantoic fluids indicated infection, and the EID_{50} (50% egg infectious dose) was calculated by the method of Reed and Muench(9). The hemagglutination test was done by the spot plate method(10) or according to the method of Salk(11) when quantitative data were desired. *Embryonated egg tests.* Groups of 8-day-old embryonated chicken eggs were infected by the allantoic route with approximately 100 EID_{50} of mumps virus diluted in antibiotic broth. After an incubationary period of 6 days at 36°C the number of infected embryos was determined by the presence of hemagglutinins in the allantoic fluids. Influenza B virus, Influenza A virus or NDV diluted in antibiotic broth to 100 LD_{50} (50% lethal dose) was injected into the allantoic sac of 10-day-old chicken embryos. The eggs were incubated at 36°C for 10 days and embryonic deaths were recorded daily. Each test and control group consisted of 8 to 14 fertile eggs. One hour after infection with either virus, or as indicated in the text, 0.5 ml of the test material was inoculated by the allantoic route. The control groups received 0.5 ml of antibiotic broth. *De-embryonation technic.* The combined *in ovo-de-embryonation* technic as described by Finter, *et al.*(12) was employed.

Results. A. Effect of algal polysaccharides on viral infections in chicken embryos. The algal polysaccharides at a dose of 1.25 mg/embryo suppressed multiplication of mumps virus in 70-80% of the infected embryos and protected 70-90% of the embryos against death from influenza B virus infections (Table I). On the other hand, no protection was demonstrable under identical experimental conditions against infections with NDV and 2 strains of influenza A virus. Similar results were obtained when treatment preceded the infection by 1 hour or 24 hours. None of the polysaccharide-treated uninfected control embryos died and all hatched normally. Furthermore, histological examination of the chorioallantoic membranes showed no microscopic changes as a result of treatment by either polysaccharide.

Dose response. To determine minimum effective dose of polysaccharide, varying amounts of both polysaccharides were injected at doses ranging from 5 to 1280 mcg/fertile egg infected with influenza B virus. As shown in Fig. 1, 40 mcg carrageenan extract/embryo gave almost maximum protection (about 80%), whereas 5 mcg protected about 30% of the embryos. The results obtained with preparations of *Gelidium cartilagenium* were similar in the region of higher dose levels but little or no activity was found when less than 80 mcg was employed. Using 10-fold dilution of influenza B virus, the results, summarized in Table II, show that 1000 times

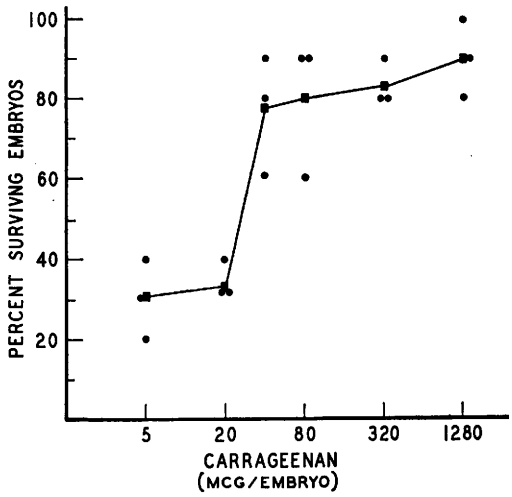

FIG. 1. Effect of varying amounts of carrageenan extract on chicken embryos infected with 100 LD_{50} of influenza B virus. Curve represents arithmetic mean of 3 experiments.

TABLE II. Infectivity Titration of Influenza B Virus in Chicken Embryos Treated with Carrageenan Extract (Treatment and Infection by the Allantoic Route).

Virus dilutions	Control embryos,* S/T	Treated embryos,† S/T
10^{-4}	0/10	1/10
10^{-5}	"	5/10
10^{-6}	"	9/10
10^{-7}	"	10/10
10^{-8}	5/10	"
10^{-9}	10/10	"
50% virus titration end point	$10^{-8.0}$	$10^{-5.0}$

* 0.5 ml antibiotic broth 1 hr after infection.
† 78 μg carrageenan extract 1 hr after infection.

more virus was required to cause a 50% fatal infection in embryos treated with 78 mcg carrageenan extract than in controls.

Effect of delayed treatment: Groups of 14 randomly selected embryonated eggs infected with influenza B virus were treated at 1, 18 or 24 hours after infection with 40 mcg/egg of carrageenan extract. Four eggs from each group were used for repeated withdrawal of 0.2 ml allantoic fluid at various intervals up to 72 hours, and the amount of virus in these fluids was determined by egg infectivity titration (Fig. 2). Multiplication of virus in the 14 controls began after a lag period of 6 hours and viral concentration reached a peak titer of $10^{9.3}$ at 48 hours. On the other hand, treatment with polysaccharide caused a temporary suppression in viral multiplication for 48 hours; this was most marked when treatment had been started 1 hour after infection. All embryos of the control group died between 4 and 5 days after infection, whereas 40 to 80% of treated embryos survived. Similar results were obtained in experiments with extracts of *Gelidium cartilagenium*. In an identical experiment using the PR8 strain of influenza A virus, no significant differences in viral titers were noted in the treated and control groups, and none of the embryos survived the 10-day period of observation.

Effect of treatment through the yolk sac. Groups of 7-day-old chicken embryos were injected into the yolk sac with 1.25 mg algal polysaccharides. After 1 hour or after 3 days 100 LD_{50} of influenza B virus was injected into the allantoic sac. No antiviral effect was demonstrable in two such experiments.

B. In vitro effects. Effects on viral infectivity: For this study the carrageenan or *Gelidium* extracts were dissolved at a dose of 80 mcg in 4.5 ml of allantoic fluid containing 100 LD_{50}/ml of influenza B virus, the mixtures were incubated at 36°C, and aliquots were removed for viral infectivity titration at 0, 1 and 4 hours. Since each test material showed the same viral concentration as the control specimens without polysaccharide, we concluded that these polysaccharides exerted no virustatic or virucidal effect *in vitro*.

Effects on hemagglutination. Green and Woolley(6) suggested that with influenza A a correlation exists between the inhibition of multiplication and of hemagglutination by polysaccharides. To examine this problem the effects of these algal polysaccharides were studied both on the chicken red blood cell as well as on the viruses (NDV, mumps, and the WS, PR8 and strains of influenza) themselves. 0.3 ml of packed chicken red blood cells was added to 2 ml of saline containing 1280 mcg of polysaccharides. The mixture was incubated at room temperature for 3 hours with frequent agitation. The cell suspension was centrifuged, washed 3 times with saline, and then resuspended in saline to 0.5% concentration. Agglutination did not occur at any

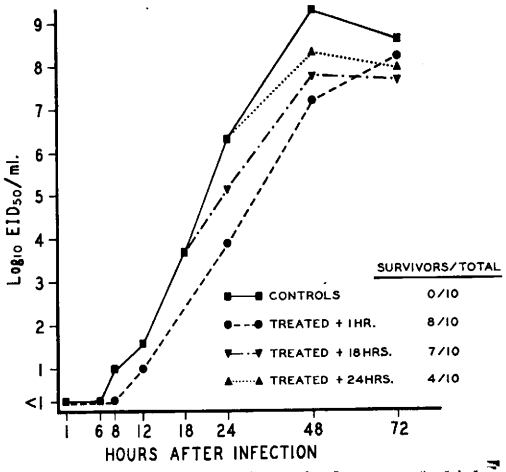

FIG. 2. Virus content and survival rates of chicken embryos treated with 40 μg of carrageenan at various intervals following infection with 100 LD_{50} of influenza B virus. (Each point represents geometric mean of viral titers of 4 individual allantoic fluid samples.)

time in the absence of virus, but these polysaccharide-treated cells were agglutinated by 4 agglutinating units of each virus. Two-fold serial dilutions ranging from 1280 to 10 mcg of each extract/0.25 ml of saline were prepared and added to an equal volume of 4 agglutinating units of each virus in saline. The mixtures were held at room temperature for one hour before adding 0.5 ml of a 0.5% suspension of chicken red blood cells. No inhibition of hemagglutination was noted at any concentration of the polysaccharides.

Horsfall and McCarty(4) reported a modifying effect of intranasally administered agar upon intranasal infections with pneumonia virus of mice (PVM). A limited study was therefore performed with these algal polysaccharides in 3- to 4-week-old albino Swiss mice which had been infected intranasally with 100 LD_{50}'s of PVM or of influenza viruses (PR8 and Lee strain). Intranasal instillations of 60 mcg of polysaccharide per mouse, given 24 hours before or up to 4 hours after infection with lethal doses of PVM, protected 70% of the animals, but had no effect upon the course of infections with influenza A or B. Furthermore, no modifying effect was observed with either polysaccharide given intravenously or intraperitoneally.

Discussion. The antiviral effects of polysaccharide-containing substances extracted from *Gelidium cartilagenium* or carrageenan on chicken embryos were rather specific. These agents were non-toxic in the concentrations employed, and caused a transient suppression of growth of influenza B virus and a reduction of the infectivity of mumps virus; they were, however, without effect upon influenza A virus or NDV. The results obtained suggest the possibility that these polysaccharides may act intracellularly.

Ginsberg(5) postulated that PVM and mumps viruses require metabolic systems different from NDV and influenza virus, because polysaccharide derived from *Klebsiella pneumoniae* selectively inhibited only the former pair. Since the results of this study have demonstrated that extracts of *Gelidium cartilagenium* and carrageenan selectively interfere with the multiplication of PVM, mumps and influenza B virus, it would appear that these viruses may share metabolic pathways which can be blocked by certain polysaccharides.

Other algal polysaccharides, such as laminarin, fucoidin and algin, which lack galactan units in their chemical composition, showed no antiviral properties(6,13).

Summary. 1) Polysaccharides extracted from *Gelidium cartilagenium* and carrageenin exerted a marked inhibitory effect on growth of influenza B virus and mumps virus in embryonated eggs but had no effect on the multiplication of influenza A virus and NDV. Significant protection against otherwise fatal infections with influenza B virus was obtained when treatment was delayed as long as 24 hours after viral inoculation. 2) Use of commercial agar as a vehicle for insoluble compounds may yield misleading results in screening tests in chicken embryos infected with either influenza B or mumps virus. The use of inert agents such as carboxymethyl cellulose obviates this difficulty.

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