TERATOLOGY 23:273-278 (1981)

Teratogenic Effect of Lambda-Carrageenan on the Chick Embryo

BENITO MONIS AND ROBERTO A. ROVASIO
I°Cátedra de Histología, Embriología y Genética, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba 5000, Argentina

ABSTRACT Carrageenans are widely used as food additives. Thus, it seemed of interest to test their possible teratogenic action. For this purpose, 530 chick eggs were injected in the yolk sac with 0.1 ml of a solution of 0.1% lambda-carrageenan in 0.9% sodium chloride. As controls, 286 eggs were injected with 0.1 ml of 9.0% sodium chloride. In addition, 284 eggs received no treatment. After incubation for 48–50 hours at 39°C, embryos were fixed, cleared, and observed with a stereoscopic microscope.

The frequency of abnormal embryos in the group receiving lambda-carrageenan was higher than in the controls ($p < 0.04$). Partial duplication of the body, abnormal flexures of the trunk, anencephaly, a severely malformed brain, thickening of the neural tube wall, an irregular neural tube lumen with segmentary occlusion and a reduction in crown-rump length and number of somites were distinctly seen in the lambda-carrageenan-injected group. Moreover, the average number of anomalies per embryo in the lambda-carrageenan-injected group was nearly twice that in the controls. Present data indicate that lambda-carrageenan has teratogenic effects on early stages of the development of the chick embryo.

Carrageenans are polysaccharides obtained from red seaweeds. They are linear polymers formed by galactose units, having various degrees of sulfation (Percival, '72). Native carrageenan has been fractionated into kappa- and lambda-carrageenan, which differ in physical properties and chemical structure. Carrageenans are widely used in the food industry as additives because of their thickening, gelling, and stabilizing properties (Tarr, '55). Therefore, it seemed of interest to establish whether lambda-carrageenan has a teratogenic action on an early period of development of the chick embryo.

MATERIALS AND METHODS

One thousand one hundred fertile chick eggs of a hybrid Pilch line were used in the present study. They were obtained from a commercial hatchery, subjected to disinfection with formaldehyde (McLaughlin et al., '63), and stored at 10°C for no more than 4 days. The study was performed with lots of 50–100 eggs every 30–45 days. Prior to incubation, the yolk sacs of 530 unopened eggs were injected with 0.1 ml of a sterile solution of the 0.1% lambda-carrageenan (Marine Colloids, Inc., Rockland, Maine) in 0.9% sodium chloride (299 mOsm/1; pH 6.20). For controls, 284 eggs received no treatment, whereas 286 eggs were injected with 0.1 ml of sterile 0.9% sodium chloride (300 mOsm/1; pH 6.20). For this injection, after cleaning with 70% ethanol, the shell was pierced near the equator with a dental drill in order to introduce a 20-mm length hypodermic needle through which the corresponding solution was injected. The needle was then gently removed and the hole covered with a piece of adhesive tape. Evidence that the injected solutions reached the yolk sac was obtained by testing the distribution of 0.1 ml of 1% India ink or 0.05 ml of the carrageenan solution mixed with 0.05 ml of 2% alcian blue in 0.1 M HCl (pH 1.0) in an identical manner. The eggs were boiled and the location of the dye was determined after transverse sectioning of the eggs.

All three groups of eggs were then incubated for 48–50 hours at 39°C. At this time, embryos were promptly removed and fixed in Bouin's fluid for 12 hours, dehydrated in graded alcohols, cleared in cedar oil, and studied under a stereoscopic microscope. At the time of removing the embryos no development was noted in 26 of the 530 eggs injected with lambda-carra-

Received March 24, 1980; accepted October 6, 1980.

0040-3709/81/2302-0273$02.00

TABLE 1. Frequency of abnormal embryos in lambda-carrageenan-injected and control groups

Experimental group	Number of embryos		P[1]
	Normal	Abnormal	
Lambda-carrageenan-injected	348	156	<0.04
Saline-injected	207	65	N.S.
Nontreated	208	63	

[1]Chi-square test with Yates' correction.
N.S. = Not significant.

geenan; 14 of the 286 injected with the sodium chloride solution; and 13 of the 284 noninjected eggs. The remainder were alive, as shown by the observation of heartbeats. Normality was based on Hamburger and Hamilton's series ('51). In 140 embryos which showed no anomalies, the crown-rump length was measured using a micrometric ocular device, and the number of somite pairs was determined. For 90% confidence limits, a range covering a mean of ±1.65 SD (Bancroft, '57), the normal values were: crown-rump length 5.14 ± 1.4 mm, and the number of pairs of somites was 19.9 ± 4.1. The mean value of the number of somite pairs herein reported is in agreement with Hamburger and Hamilton's data ('51) corresponding to stages 12–15 and an incubation time of 48–50 hours. The significance of the frequency of malformed embryos and of the various abnormalities that were encountered was determined using the Chi-square test with Yates' correction for continuity. For quantitative data, the variation coefficient was determined and the test for significance of means was done.

RESULTS

Abnormal embryos were found in both carrageenan-injected and control groups. The frequency of abnormal embryos in the group of eggs injected with lambda-carrageenan (156/504) was significantly higher than in the controls (128/543; Table 1). No significant difference was noted in the number of abnormal embryos in the group injected with the saline solution and the noninjected group.

Various types of anomalies were recognized in both experimental and control groups; these included partial duplication of the cephalic or caudal end of the embryo, or both (Fig. 1); reduction of the crown-rump length (Figs. 2 and 3); diminution of the number of somites (Fig. 3); abnormal flexures (Fig. 4); subnormal development of the brain (Figs. 2–4); anencephaly (Fig. 5); and severe malformations of the brain (Fig. 6). In some cases, deformities and bending of the spinal cord were also recorded. There was focal thickening of the wall of the neural tube with segmentary occlusion of the ependymal duct (Figs. 7 and 11). On occasion, the ependymal lumen was hourglass in shape (Fig. 2) or, more often, absent, irregular, or reduced in size (Figs. 8–10). Saccular dilatations, bending of the wall of the neural tube (Fig. 12), and a third row of somites were also seen (Fig. 13). Additionally, severe disorganization of the caudal end of the neural tube was found (Fig. 14).

The percent frequency and the significance of the various anomalies in experimental and both control groups are shown in Table 2. A highly significant statistical difference was noted between the control and carrageenan-treated groups with respect to segmental occlusions of the spinal cord lumen ($p < 0.004$). For other anomalies, such as coarse malformations of the brain, partial duplication of the body, and reduction of the crown-rump length, the frequency in the carrageenan-injected group was significant. The percentage of other malformations, such as anencephaly and decreased number of somites, was greater in the carrageenan-injected group than in the controls, even though this tendency did not reach statistical significance.

Mean values of the crown-rump length and number of somites in abnormal embryos of experimental and both control groups are recorded in Table 3. As shown, the reduction in both parameters was significantly changed in the carrageenan-injected group.

The mean number of anomalies per embryo in the carrageenan-injected group was: 3.9 ± 0.2; in the saline-injected group: 2.3 ± 0.1; and in the nontreated group: 2.1 ± 0.2 ($p < 0.001$).

DISCUSSION

Our data indicate that lambda-carrageenan, under the experimental conditions of the present study, has a teratogenic effect on chick embryos. These observations are in keeping with those of Hunt ('51), who showed that various malformations were caused by the injection of sucrose into the albumen of the chick egg. Hughes et al. ('74) reported that certain sugars (mono-, di-, and trisaccharides) can induce malformations in chick embryos. Galactose added in various concentrations to a diet fed to pregnant rats produced anophthalmia and lens abnormalities in the fetus (Bannon et al., '45; Demeyer, '59). Addition of D-glucose to the cul ture medium produced teratogenic effects on rat embryos (Cockroft and Coppola, '77). Also, malformations of the brain and heart have

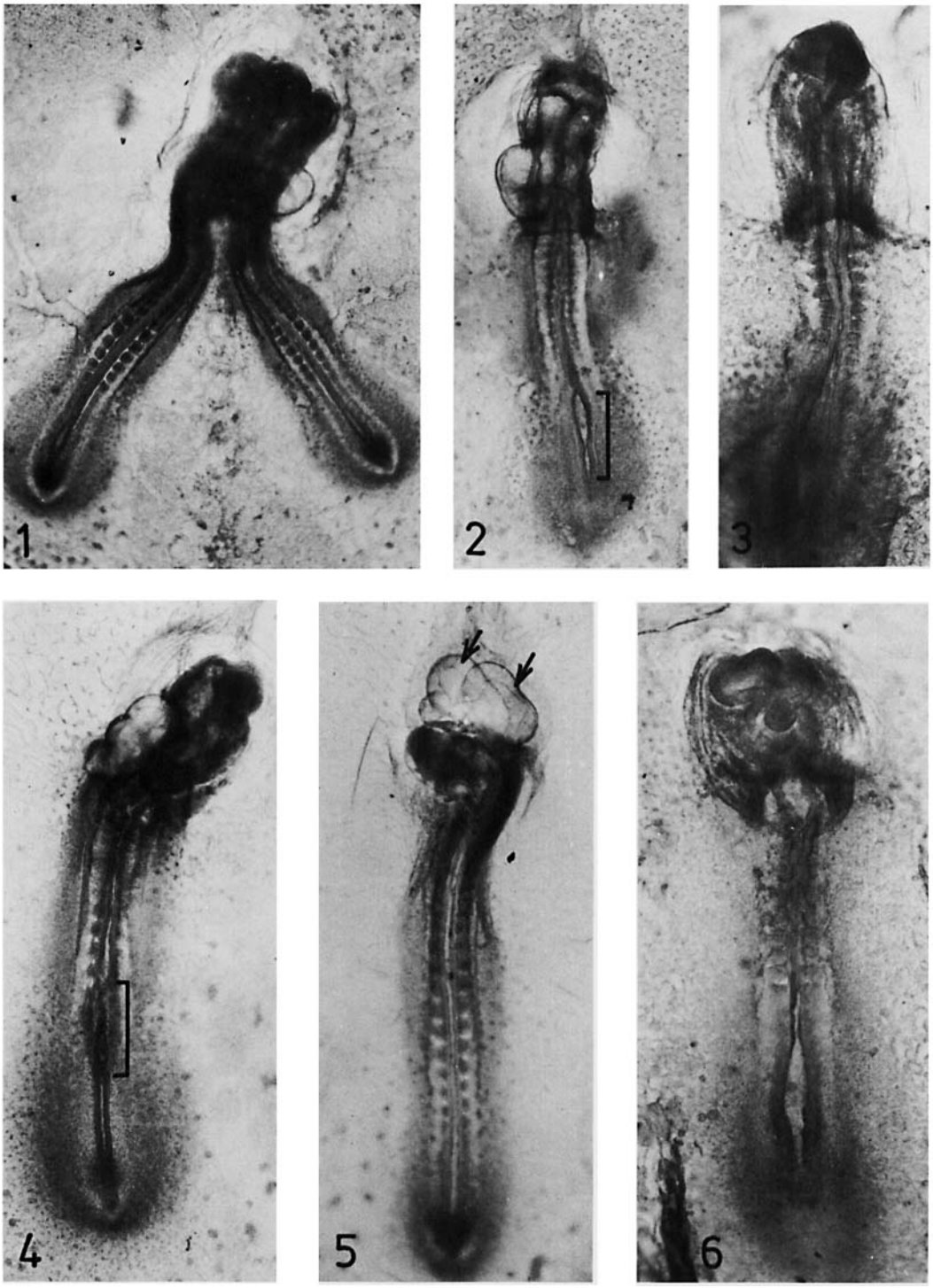

Fig. 1. All pictures correspond to abnormal embryos of the lambda-carrageenan-injected group. (1) Partial duplication of the trunk and caudal end. × 17. (2) Deformed and underdeveloped forebrain and hourglass-shaped neural tube lumen (bracket). × 21. (3) Grossly deformed primitive brain, incomplete closure of the neural tube and diminution of the number of somites. × 35. (4) Abnormal cervical flexure and disorganization of the spinal cord. There is probably lack of closure of the neural tube, whereas the somites are poorly developed (bracket). × 20. (5) Anencephalic embryo with heart duplication (arrows). × 30. (6) Poorly developed embryo showing a relatively enlarged cephalic end corresponding to a grossly malformed brain. × 40.

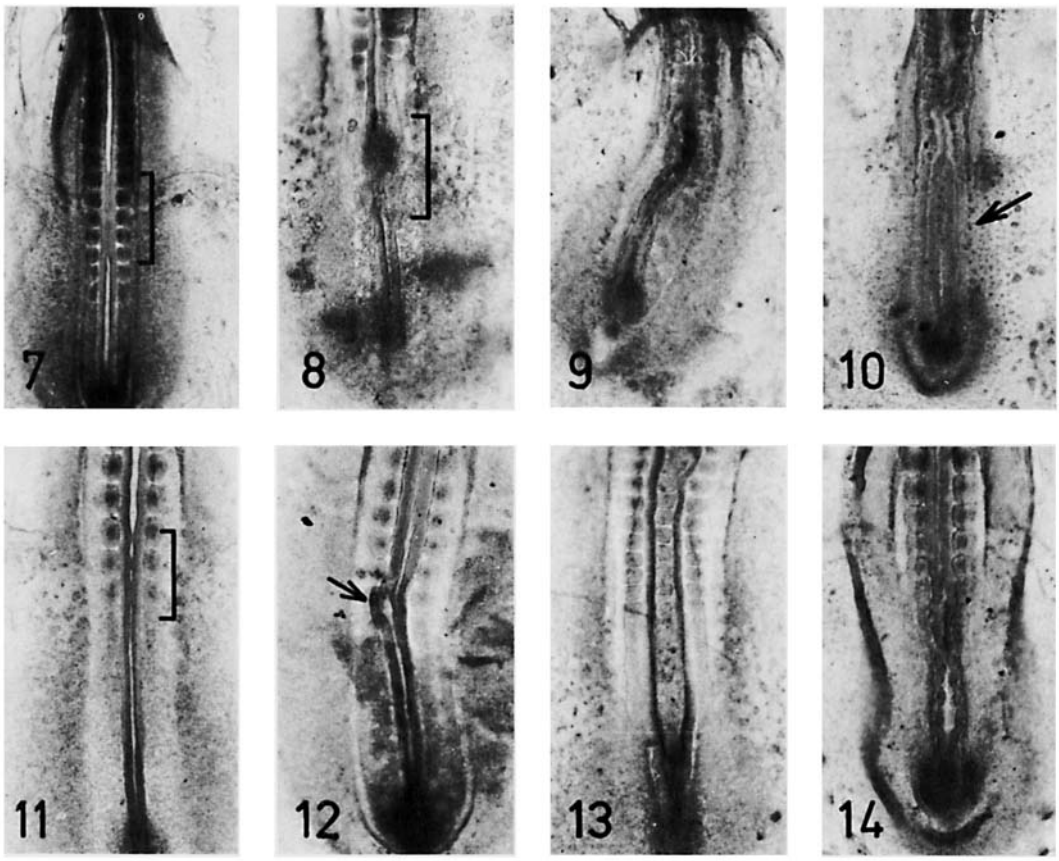

Fig. 2. All pictures correspond to abnormal embryos of the lambda-carrageenan-injected group. (7) Thickening of the neural tube wall and segmentary occlusion of the lumen (bracket). × 20. (8) Neural tube markedly distorted by a tissue mass (bracket). In the adjacent mesenchyme, somites are not seen. × 25. (9) Multiple flexures of the embryo axis with focal occlusion of the neural tube. × 25. (10) The neural tube, which has a wavy appearance, is probably open (arrow). × 18. (11) Multiple segmentary occlusions of the lumen of the neural tube (bracket). × 35. (12) Deformity and angulation of the embryo axis, with saccular dilatation of the neural tube (arrow). × 35. (13) The neural tube is probably open. There is a medial, third row of somites. × 34. (14) Deformities and disorganization of the caudal end of the neural tube. × 28.

been shown in fetuses of diabetic rats (Deuchar, '77). The lack of teratogenic effects of carrageenan in intact mammals has been reported (Collins et al., '77, '79).

The anomalies that followed carrageenan administration to chick embryos were similar to those induced by a large number of teratogenic agents. Romanoff ('72), quoted by Hughes et al. ('74), listed 74 substances, many of which caused malformations of the neural tube and tail region of chick embryos incubated for 0–48 hours.

The present results support the view that the neural tube and the embryonic axis are highly sensitive to various teratogenic agents at relatively early stages of development (Hughes et al., '74). Furthermore, since lambda carrageenan elicited abnormalities similar or identical to those induced by many other agents, both chemical and physical, it can be suggested that a common mechanism underlies the pathogenesis for most if not all of them.

At least two mechanisms can be postulated for the teratogenic effect of lambda-carrageenan on the chick embryo: (1) carrageenan may bind to cell surfaces, interfering with normal morphogenesis; or (2) this polygalactoside may be degraded to simpler sugars such as galactose, which has teratogenic properties (Shepard, '73; Hughes et al., '74). There are data in

TABLE 2. Percentage of anomalies in lambda-carrageenan-injected and control groups

Malformation	Lambda-carrageenan-injected (280)[1]	Saline-injected (272)[1]	P[2]	Nontreated[3] (118)[1]
Partial duplication of the body	2.50 (7)[4]	0.37 (1)[4]	< 0.03	0.00 (0)[4]
Severe malformation of the brain	3.90 (11)	0.73 (2)	< 0.015	0.00 (0)
Anencephaly	3.20 (9)	0.73 (2)	N.S.	0.00 (0)
Segmental occlusion of the spinal cord	9.60 (27)	3.31 (9)	< 0.004	2.54 (3)
Reduction of the crown-rump length	18.20 (51)	11.76 (32)	< 0.05	9.32 (11)
Diminution of the number of somites	18.60 (52)	13.23 (36)	N.S.	10.17 (12)

[1]Number of embryos taken at random from each experimental group.
[2]Chi-square and G tests with Yates' correction.
[3]All corresponding values for saline-injected and nontreated eggs were not significant.
[4]Number of abnormal embryos.
N.S. = Not significant.

TABLE 3. Mean values of the crown-rump length and of the number of somites in abnormal embryos of lambda-carrageenan-injected and control groups

Experimental group	Number[1]	Crown-rump length (mm)[2]	P[3]	Number of somite pairs[2]	P[3]
Lambda-carrageenan-injected	77	3.3 ± 0.1		11.8 ● 0.8	
			< 0.01		< 0.05
Saline-injected	27	3.8 ± 0.1		14.8 ± 1.0	
			N.S.		N.S.
Nontreated	38	4.1 ± 0.2		14.0 ± 1.2	

[1]Abnormal embryos taken at random from each experimental group.
[2]Mean ± SE.
[3]Text of the differences of means.
N.S. = Not significant.

support of both hypotheses. Concerning a direct effect of carrageenan on cell surfaces, it can be mentioned that cell-surface components play a role in cell-to-cell interactions and embryonic morphogenesis (McLean, '77; Moscona and Hausman, '77). On the other hand, it has been reported that erythrocytes may bind sulfated polysaccharides including carrageenan (Pittz et al., '77 a,b). There are data indicating that the glycosylation of the terminal end of the corresponding sugar chain will be catalyzed by surface glycosyltransferases (Waechter and Lennarz, '76; Shur, '77 a,b). It is possible that carrageenan may interfere with the coupling of the terminal sugars of cell-surface glycoconjugates.

With regard to a mechanism mediated by products of carrageenan degradation in vivo, strong acid phosphatase activity has been shown in the chick blastoderm edge, with distinct enzymatic activity in yolk globules, suggesting that the latter correspond to lysophagosome structures (Beck, '65). Furthermore, it has been shown that carrageenan is secluded by macrophages in secondary lysosomes (Monis et al., '68; Monis and Valentich, '73; Abraham et al., '74; Thompson et al., '76), in keeping with the finding of increased alpha- and beta-D-galactosidase activities of a lysosomal fraction obtained from a macrophage-rich granuloma induced by the subcutaneous injection of carrageenan in the rat (Valentich and Monis, '74). It is possible that breakdown of carrageenan in the embryo or in cells of the area vasculosa, or both, releases galactose, which caused the anomalies herein reported.

ACKNOWLEDGMENTS

The authors are indebted to Lic. Federico Kalinec and Liliana Orellana de Urena for the statistical analysis of the experimental data.

This work was supported by CONICET, Argentina.

LITERATURE CITED

Abraham, R., R.J. Fabian, L. Golberg, and F. Coulston (1974) Role of lysosomes in carrageenan-induced cecal ulceration. Gastroenterology, *67:* 1169–1181.

Bancroft, H. (1957) Introduction to Biostatistics. Hoeber-Harper, New York.

Bannon, S.L., R.M. Higginbottom, J.M. McConnell, and H.W. Kaan (1945) Development of galactose cataract in the albino rat embryo. Arch. Ophthalmol., *33:* 224–228.

Beck, F. (1965) The distribution of acid phosphatase in the chick blastoderm. Exp. Cell Res., *37:* 504-508.

Cockroft, D.L., and P.T. Coppola (1977) Teratogenic effects of excess glucose on head-fold rat embryos in culture. Teratology, *16:* 141-146.

Collins, T.F.X., T.N. Black, and J.H. Prew (1977) Long-term effects of calcium carrageenan in rats. II. Effects on foetal development. Food Cosmet. Toxicol. *15:* 539-545.

Collins, T.F.X., T.N. Black, and J.H. Prew (1979) Effect of calcium and sodium carrageenans and i-carrageenan on hamster foetal development. Food Cosmet. Toxicol. *17:* 443-449.

Demeyer, R. (1959) Action teratogene du galactose administré a la rate gravide. Ann. Endocrinol. (Paris), *20:* 203-211.

Deuchar, E.M. (1977) Embryonic malformations in rats, resulting from maternal diabetes: preliminary observations. J. Embryol. Exp. Morph., *41:* 93-99.

Hamburger, V., and H.L. Hamilton (1951) A series of normal stages in the development of the chick embryo. J. Morphol., *88:* 49-92.

Hughes, A.F., R.B. Freeman, and T. Fadem (1974) The teratogenic effects of sugars on the chick embryo. J. Embryol. Exp. Morphol., *32:* 661-674.

Hunt, E.L. (1951) Developmental abnormalities in chick embryos treated with sugar. Science, *113:* 386-387.

McLaughlin, J. Jr., J.-P. Marliac, M.J. Verret, M.K. Mutchler, and O.G. Fitzhugh (1963) The injection of chemicals into the yolk sac of fertile eggs prior to incubation as a toxicity test. Toxicol. Appl. Pharmacol. *5:* 760-771.

McLean, R.J. (1977) Membrane specialization in the course of differentiation. In: Mammalian Cell Membranes. G.A. Jamieson and D.M. Robinson, eds. Butterworths, London-Boston, Vol. 3, pp. 250-265.

Monis, B., T. Weinberg, and G.J. Spector (1968) The carrageenan granuloma in the rat. A model for the study of the structure and function of macrophages. Br. J. Exp. Pathol., *49:* 302-310.

Monis, B., and M.A. Valentich (1973) Secondary lysosomes of macrophages. Structure and Biochemistry. Acta Physiol. Lat. Am., *23:* 255-257.

Moscona, A.A., and R.E. Hausman (1977) Biological and biochemical studies on embryonic cell-cell recognition. In: Cell and Tissue Interactions. J.W. Lash and M.M. Burger, eds. Raven Press, New York, pp. 173-185.

Percival, E. (1972) Chemistry of agaroids, carrageenans and furcellarians. J. Sci. Food Agric., *23:* 933-940.

Pittz, E.P., R. Jones, L. Golberg, and F. Coulston (1977a) Interaction of polysaccharides with plasma membranes. I. Interaction of human erythrocytes with degraded iota carrageenans and the effect of dextran and deae dextran. Biorheology, *14:* 21-31.

Pittz, E.P., R. Jones, L. Goldberg, and F. Coulston (1977b) Interaction of polysaccharides with plasma membranes. II. Effects of ionic strength, temperature and trypsin digestion of the erythrocyte surface on the aggregation of erythrocyte by iota carrageenans. Biorheology, *14:* 33-42.

Romanoff, A.L. (1972) Pathogenesis of the Avian Embryo. Wiley-Interscience, London, pp. 243-279.

Shepard, T.H. (1973) Catalogue of Teratogenic Agents. The John Hopkins University Press, Baltimore and London, pp. 1-211.

Shur, B.D. (1977a) Cell-surface glycosyltransferases in gastrulating chick embryos. I. Temporally and spatially specific pattern of four endogenous glycosyltransferase activities. Dev. Biol., *58:* 23-39.

Shur, B.D. (1977b) Cell-surface glycosyltransferases in gastrulating chick embryos. II. Biochemical evidence for a surface localization of endogenous glycosyltransferase activites. Dev. Biol., *58:* 40-55.

Tarr, H.L.A. (1955) Sea foods. In: Handbook of Food and Agriculture. F.C. Blank ed., Reinhold, New York, pp. 511-531.

Thompson, A.W., A.R. Wilson, W.J. Cruichshank, and A.H. Jeffries (1976) Evidence for a selective cytotoxic effect of carageenan on cells of the immune system in vivo and in vitro. Experientia, *32:* 525-526.

Valentich, M.A., and B. Monis (1974) Glycosidases activities of a carrageenan granuloma. Fed. Proc., *33:* 608.

Waechter, C.J. and W.J. Lennarz (1976) The role of polyprenol-linked sugars in glycoprotein synthesis. Annu. Rev. Biochem., *45:* 95-112.