Short Communication

The Incorporation of $^{35}$S-Labeled Sulfate into Carrageenan in Chondrus crispus

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During an investigation of carbohydrate metabolism in red algae, we noted that freshly harvested Chondrus crispus resuspended in a defined salts medium containing $^{35}$SO$_4^-$ rapidly removes sulfate from the external medium and incorporates it into carrageenan (3, 5, 7, 8, 11, 12). An account of this observation is presented here.

MATERIALS AND METHODS

C. crispus was harvested during August, 1970 in the vicinity of Nobbska Point, Cape Cod, Massachusetts and was held in flowing seawater until used, in no instance over 18 hr. Selected fronds, free of epiphytes, were cut into 0.1-g pieces, rinsed with sterile-filtered ASP${}_m$ medium (9) in which MgSO$_4$ had been replaced with MgCl$_2$, and transferred to a sterile flask containing sterile medium of the same composition supplemented with trace elements in the form of salts other than sulfate. The chemical and radioactive level of sulfate in each flask was determined by appropriate additions of Na$^{14}$SO$_4$ and carrier-free H$^{35}$SO$_4$. Traces of sulfate present in the medium prior to addition of carrier-free H$^{35}$SO$_4$ was estimated as 0.1 $\mu$M.

In a typical experiment, 10 pieces of seaweed were suspended in 20 ml of medium containing $^{35}$SO$_4^-$ (5 $\mu$Ci) in a 50-ml Erlenmeyer flask. Flasks were shaken in a thermostatted gyratory shaker (18 C) under cool-white fluorescent light (500 ft-c). At intervals, aliquots of medium were removed and counted in Bray's solution in a liquid scintillation spectrometer. In some experiments, pieces of seaweed were also analyzed for incorporation of $^{35}$S into water-extractable polysaccharides.

Carrageenan was recovered from the labeled seaweed by extracting the ground-up tissue with boiling water for 24 hr. The insoluble residue was removed by centrifugation (17,000g; 20 min) and solid KCl (0.12 g/ml) was added with stirring to the clear supernatant while maintaining the solution at 50 C. On standing at 25 C, a finely divided gel settled out and was pelleted by centrifugation. It occupied one-tenth of the original volume of the extract. The pellet, resuspended in fresh 0.15 $\mu$M KCl, was stirred for 4 hr to allow any remaining KCl-soluble labeled polysaccharide to leach from the gel. The gel was recovered by centrifugation, dissolved in hot water (5 volumes, 80 C) and converted to its Na$^+$ salt by passing the solution through a column of Dowex 50W-X8 resin (Na$^+$ form). Effluent and washes were combined and lyophilized.

Gel filtration of KCl-soluble and KCl-insoluble polysaccharides was accomplished on a column of Sephadex G-100 gel (42 x 1.2 cm, flow rate 6 ml/hr). Fractions were analyzed for carbohydrate (as galactose) with anthrone reagent (6) and for $^{35}$S by liquid scintillation counting.

RESULTS AND DISCUSSION

When incubated under conditions described in the preceding section, about 50% of the $^{35}$S from a medium containing only "carrier-free" sulfate was taken up by C. crispus in the first 12 hr (Fig. 1). At a sulfate concentration of 10 $\mu$M or lower, over 80% of the label present in the medium was removed by the seaweed in 22 hr. A somewhat lower value of $^{35}$S present in tissue incubated for 28 hr probably reflects release of labeled sulfated polysaccharide into the medium. If a higher concentration was provided, label continued to accumulate in the seaweed throughout the 28-hr period of the experiment although the rate at which it was taken up diminished, noticeably so at 1 mM. When incubated in medium containing 0.1-1.0 mM sulfate, uptake of that salt amounted to about 0.5% of the seaweed present (on a dry weight basis) in the first 20 hr. To determine how much of the $^{35}$S had been incorporated into carrageenan, an air-dried sample of seaweed that had been incubated for 28 hr in medium containing labeled 0.1 mM sulfate was pulverized and extracted with boiling water (170 mg in 25 ml) for 24 hr. Of the total $^{35}$S present in the dry seaweed, 92% was recovered in the clear supernatant after centrifugation. Addition of solid KCl to make the final solution approximately 0.15 $\mu$M precipitated a gel containing 35% of the radioactivity (k-fraction or crude k-carrageenan). The precipitated gel was washed with fresh 0.15 $\mu$M KCl and lyophilized (68 mg, 21% as galactose by anthrone assay). An undetermined amount of KCl was also present in this k-fraction.

Dialysis of the supernatant portion of the KCl step removed

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**FIG. 1.** Incorporation of $^{35}$S-labeled sulfate into *C. crispus* as measured by uptake from the medium. The concentration of sulfate used in each experiment is recorded alongside the appropriate symbol. Data reported at 0.1 mm sulfate represents two experiments run 7 days apart.

**FIG. 2.** Separation of $\kappa$-fraction and $\lambda$-fraction and their chemically modified products on Sephadex G-100. See text for details. In all four plots presented here, the solid line (---) indicates the profile of radioactivity in eluted fractions and the broken line (X—-X) anthrone-reactive material in the same fractions. A: $\kappa$-Fraction and desulfated $\kappa$-fraction. The dotted line with open circles (O—-O) indicates the profile of radioactivity in eluted fractions of desulfated $\kappa$-fraction and the dotted line with open squares (□—-□) the anthrone-reactive material in the same fractions. B: $\kappa$-Fraction after treatment with borohydride and alkali. C: $\lambda$-Fraction. D: $\lambda$-Fraction after treatment with borohydride and alkali. The excluded volume of the column used in these separations was measured with Blue Dextran 2000 (BD) and the low molecular weight region was determined with sucrose (SUCROSE). These regions are bracketed with arrows below the abscissa.

another 25% of the label as low and intermediate molecular weight material. This fraction was not examined. The remaining KCl-soluble, high molecular weight fraction was treated with 3 volumes of ethanol to precipitate remaining polysaccharides ($\lambda$-fraction or crude $\lambda$-carrageenan). Nearly all (>90%) of the remaining radioactivity was recovered in the precipitated gel. This gel was washed with fresh ethanol-water (3:1, v/v) and lyophilized (107 mg, 30% as galactose by anthrone assay).

When these fractions were redissolved in water and placed on a gel permeation column of Sephadex G-100, elution with 0.01 M acetic acid gave results shown in Figure 2. $\kappa$-Fraction appeared as a single radioactive peak (Fig. 2A) just past the excluded volume of the column. When treated with borohydride and alkali (10), this fraction was converted to a product with a narrower radioactive peak in the same region (Fig. 2B), possibly the result of "unmasking" of repeating sequences of 1,3-linked $\beta$-D-galactosyl and 1,4-linked 3,6-anhydro-$\alpha$-D-galactosyl residues (1). Desulfation of $\kappa$-fraction by the procedure of Dolan and Rees (4) gave a product in which both the anthrone-reactive components and the radioactivity were shifted from higher to lower molecular weight regions of the profile (Fig. 2A). Most of the anthrone-reactive material appeared as a broad band of intermediate molecular weight fragments followed by a peak of low molecular weight material, and most of the radioactivity accompanied the latter. Further fractionation of this labeled low molecular weight material on Sephadex G-10 partially resolved anthrone-reactive components from $^{35}$S label, an indication that most of the ester bonds had been broken during desulfation.

When the $\lambda$-fraction was separated on Sephadex G-100, its radioactivity appeared as a broad peak after the excluded volume (Fig. 2C). Anthrone-reactive material occupied the same fractions but its profile was skewed toward low molecular weight components. After treatment with borohydride and alkali (10), the product gave a profile in which more label appeared in higher than in lower molecular weight fractions (Fig. 2D). It would appear that only a portion of the polysaccharide present in the $\lambda$-fraction responded to reductive alkali treatment, an observation one might expect in view of the heterogeneous nature of $\lambda$-fraction (12).

Infrared spectra of both fractions were made using KBr discs. Spectra of both fractions contained broad absorption bands at 1240 and 1040 cm$^{-1}$. Both also contained a narrow band at 930 cm$^{-1}$ and a broader band, notably skewed toward lower wave numbers, at 830–840 cm$^{-1}$. Similar spectra have been described by Stancioff and Stanley (13) for carrageenan fractions from *C. crispus* and other red algae.

Uptake and incorporation of $^{35}$S-labeled sulfate into fucoidin in *Fucus vesiculosus* has been described by Bidwell and Ghosh (2). This appears to be the first time a similar investigation has been extended to red algae, specifically the sulfation of carrageenan. In spite of its preliminary nature, this report promises to be of consequence regarding sulfate ester formation in *C. crispus* and in other carrageenan-rich red algae. Plants used in this study were harvested during a period of rapid vegetative growth. During this period, much carrageenan is produced. It seems likely that most of the sulfate esterified during the interval in which labeled inorganic sulfate was administered represents net synthesis of newly formed polysaccharide rather than turnover of sulfate on pre-existing carrageenan. Our observations provide a basis for testing this in future studies.

Our findings provide a simple procedure for preparing sulfate-labeled carrageenan for other purposes such as clinical and nutritional studies in which this polysaccharide is used. Further, structural studies of this polysaccharide are aided by this approach.

**SUMMARY**

Uptake and incorporation of $^{35}$S-labeled sulfate into carrageenan has been examined in *C. crispus*. Labeled sulfate appears as half-ester groups in both the KCl-soluble and KCl-insoluble fractions of the polysaccharide.
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LITERATURE CITED