BIOSYNTHESIS OF CARRAGEENANS IN HAPLOID AND DIPLOID PLANTS OF THE RED ALGA CHONDRUS CRISPUS

By

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ABSTRACT

Biosynthesis of carrageenans, the sulphated polygalactans which consitute most of the cell wall and intercellular matrix in the marine red alga Chondrus crispus, was studied using in vivo labelling with ^{35}S - sulphate and ^{14}C - bicarbonate. Pre-cultured wild-type gametophyte showed enhanced sulphate uptake and incorporation characteristic of the rapidly grown T_4 strain. It was found that sulphate uptake was related to growth status of the plants rather than to the life cycle stage (haploid gametophyte or diploid sporophyte) despite the difference in sulphation level of the major carrageenans in these different stages. An apparent K_m for sulphate uptake was determined for the T_4 gametophyte.

The sporophyte produces mainly lambda carrageenan (soluble in 0.3M KCl) while the gametophyte produces kappa carrageenan (insoluble in 0.3M KCl) and a 0.3M KCl soluble fraction very unlike lambda carrageenan (47). Lambda carrageenan consists ideally of repeating disaccharide units of 4-linked galactose - 2,6-disulphate and 3-linked galactose (70% sulphated at C2) while kappa carrageenan consists ideally of 4-linked 3,6 anhydrogalactose and 3-linked galactose-4-sulphate (68, 70).

The synthesis of lambda carrageenan was more rapid than that of kappa carrageenan. Neither molybdate nor tungstate (other Group VI anions)

inhibited sulphate incorporation into carrageenans in T_4 gametophyte nor did nitrate. NH_4Cl reduced incorporation into kappa carrageenan while urea inhibited incorporation into the 0.3M KCl soluble fraction in T_4. Plants kept in the dark showed very little incorporation of ^{14}C - bicarbonate into carrageenan. Galactose and 2,4-D showed little effect on total incorporation of ^{14}C-bicarbonate but tended to shift the labelling ot 0.3M KCl soluble and insoluble fractions.

Pulse-chase experiments showed the precursor of kappa carrageenan to be present in the 0.3M KCl soluble fraction. Subfractionation of this soluble fraction with 3M KCl followed by infrared and immunological analyses of the fractions showed a progression from iota - (4-linked 3,6 anhydrogalactose-2-sulphate and 3-linked galactose-4-sulphate) and/or nu - (4-linked galactose-2,6-disulphate and 3-linked galactose-4-sulphate) like carrageenan to kappa type with the 3M KCl soluble carrageenan being least kappa-like. A pathway for the biosynthesis of kappa carrageenan was proposed on the basis of radioisotope labelling, infrared, and immunological evidence.

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TO MY PARENTS

WITHOUT WHOSE HELP AND ENCOURAGEMENT

THIS WORK WOULD NOT HAVE BEEN POSSIBLE

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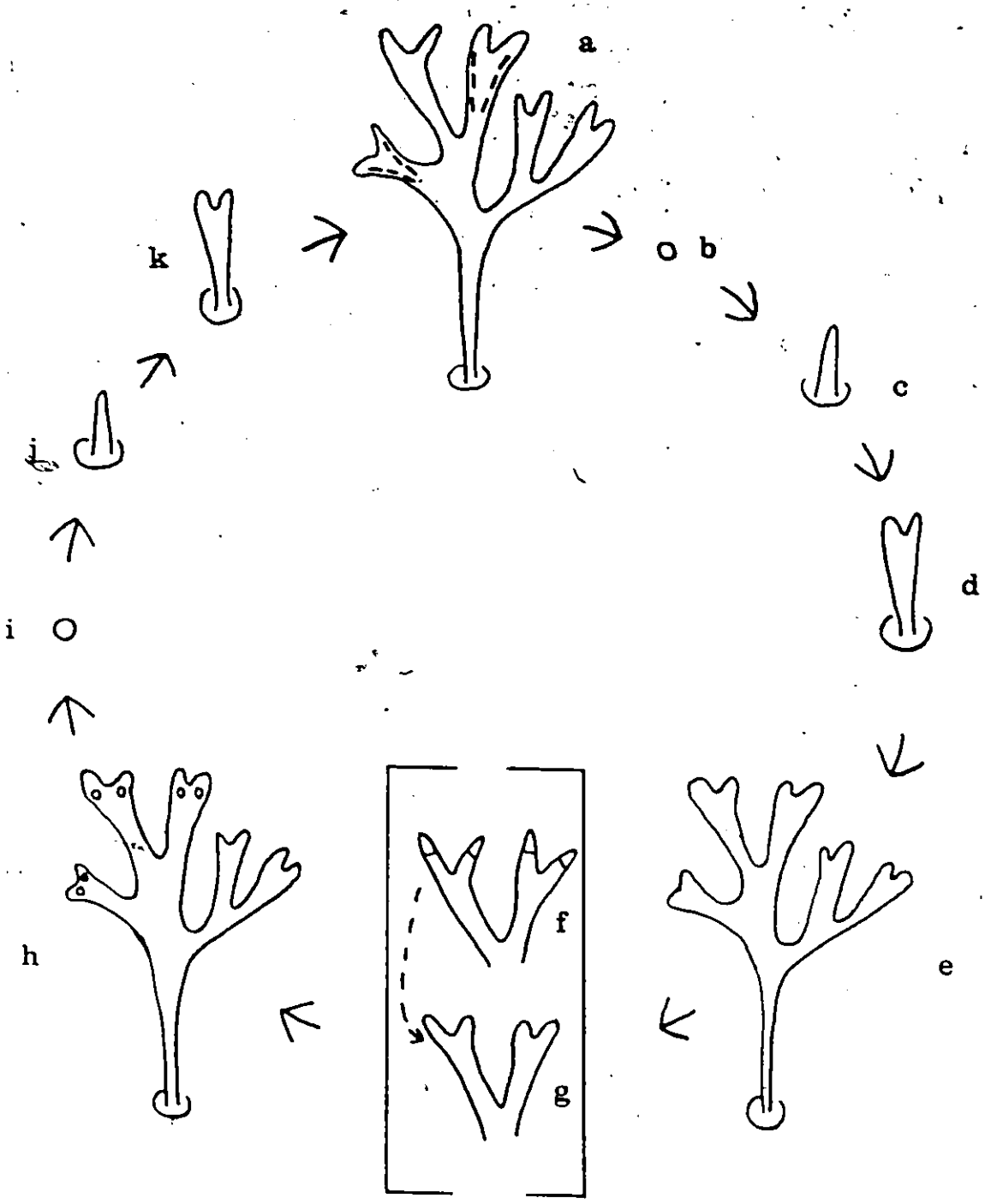
INTRODUCTION

Chondrus crispus (Stackhouse) is a marine macrophytic alga found in North America along the coastline from Labrador to New Jersey. C. crispus belongs to the class Rhodophyta, Subclass Florideae, Order Gigartinales, Family Gigartinaceae. (34) The commercial interest in C. crispus developed from the discovery of the gelling properties of certain carrageenans, major cell wall and intercellular matrix polysaccharides. These sulphated polygalactans are of considerable economic importance and scientific interest today.

Isomorphic haploid (gametophyte) and diploid (tetrasporophyte) C. crispus plants have been identified, primarily by reproductive structures. Although both karyotypes exist, an alteration of generations does not seem to be obligatory within a relatively long time period, as in certain cases plants have been propagated vegetatively, for several years in the case of T_4 gametophyte. (52).

Fig. 1 shows the different life cycle stages. The mature sporophyte produces ovate tetrasporangia maturing acropetally near apical regions (11,76). Externally tetrasporangial sori appear as elongated, oval red areas which are relatively flat but show somewhat raised areas on both sides of the frond. Internally tetrasporangial sori are recognizable by the cruciate arrangement of spores (76). Mature tetraspores give rise to gametophytic plants, both male and

Fig. 1 Life cycle of C. crispus. (a) mature tetrasporophyte (diploid) plant with sori. Tetraspores (b) presumably formed by a reduction division are released and give rise to immature gametophytic plants (c and d). The mature gametophyte (e) may grow vegetatively for long periods of time or form fruiting structures. (f) apical spermatangia of the male plant. Spermatia fertilize the female plant (g) which subsequently results in mature carposporic plants with visible sori (h). Mature carpospores (diploid) (i) are released and give rise to immature tetrasporic plants (j and k) and ultimately to mature sporophytic plants with sori (a).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female. Although fertilization has not been observed directly, carposporangial sori fail to develop on female plants cultured in the absence of male plants (11).

Male plants are hard to identify (11). They tend to be shorter and less branched than female plants of comparable age and the frond apices tend to be narrower. The main distinguishing morphological criterion for male plant identification, however, is observation of mature spermatangia as small, colourless or whitish to pinkish-white structures apically situated on male plant fronds.

The mature carposporangial sorus appears as a nearly round, apical swelling on one side of the frond with a slight concavity on the opposite side. Carposporangial sori tend to be single rather than grouped as with the tetrasporophytic sori. The diploid carposporophyte formed after fertilization has been shown to be parasitic upon the parent plant (11, 76). Mature carpospores give rise to tetrasporophytes thus completing the life cycle.

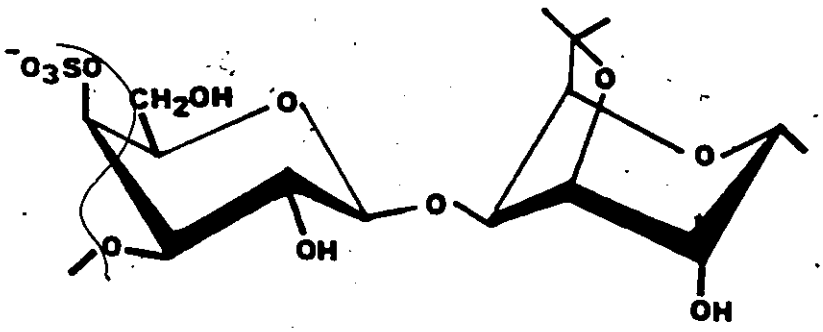
The entire life cycle has been demonstrated in culture (11), an admirable accomplishment considering the extremely slow growth rate. After 10 to 12 months in culture gametophytic plants branched not less than twice and some reached sexual maturity at a height of about 50 to 80 mm

As mentioned above, interest in C. crispus arose from early recognition of the gelling properties of C. crispus extracts (82). Until 1950, however, very little information regarding carrageenans was available . It was

known that carrageenan contained D-galactose sulphate and that it formed a strong thermoreversible gel in the presence of KCl (82). In 1953 Smith and Cook developed a method for the fractionation of carrageenan based upon KCl solubility (74). O'Neill, in 1955, demonstrated the presence of 3,6-anhydrogalactose as a major carrageenan component (57), and proposed a structure for "kappa" carrageenan (the carrageenan fraction insoluble in dilute KCl) as a linear polymer consisting of 3-linked D-galactose-4-sulphate and 4-linked 3,6-anhydrogalactose with some galactose disulphate residues (57).

Much of the chemical analysis of carrageenans was accomplished between 1960 and 1969 by Rees and his coworkers. Rees suggested a structure for carrageenans as substituted α 1-3 and β 1-4 linked galactans with repeating disaccharide structure of a particular carrageenan "masked" by some differences in substituents. (1, 70). Fig. 2 shows the idealized structures of the repeating disaccharide units in the major carrageenans. In C. crispus the KCl insoluble or kappa fraction would be defined as polymers of 3-linked β D-galactose-4-sulphate and 4-linked 3,6-anhydro α -D-galactose. The KCl soluble fraction was considered to contain two main fractions, lambda and mu carrageenans. The latter was postulated to consist ideally of 3-linked galactose-4-sulphate and 4-linked galactose-6-sulphate. Lambda carrageenan was redefined by Rees in 1969 (70) as a polysaccharide devoid of galactose-4-sulphate and 3,6-anhydrogalactose. The component saccharides were 4- linked galactose-2,6-disulphate and 3- linked galactose (70% of the 3-linked units sulphated at C2) (22, 70). The mu fraction has never been isolated but its presence was suggested by results of alkaline borohydride treatment and infrared spectra. Whether the precursor of the 3,6-anhydrogalactose unit was mono- or di-sulphated galactose was not resolved (1, 70).

Fig. 2 Idealized structures of the repeating disaccharide units in the major carrageenans. From top to bottom are those found in kappa, iota, mu and lambda carrageenans. Nu carrageenan differs from mu carrageenan in having a sulphate group on the C2 of the 4-linked unit.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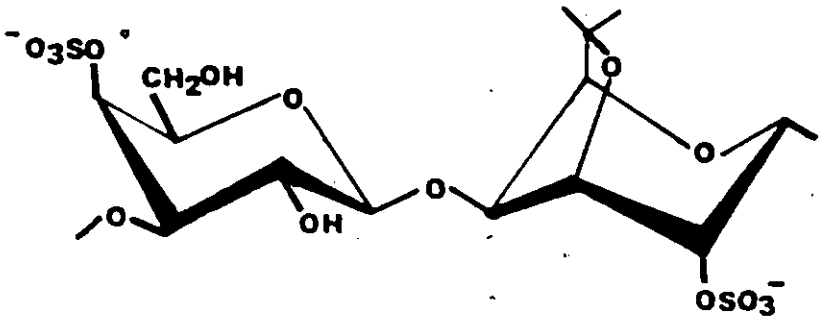
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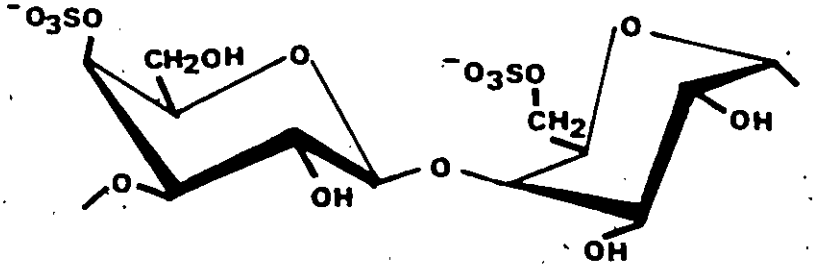
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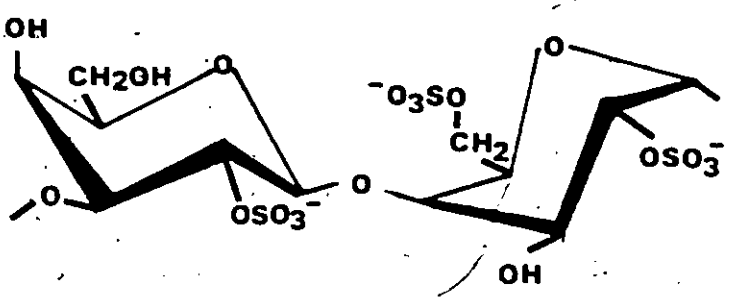
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Observed variations in the kappa/lambda ratio were attributed to seasonal or harvest location factors (including light duration, light quality, temperature, salinity, nutrient supply, and intertidal exposure) (7, 9, 26). It was not until 1972 that the source of such variations was found. At a symposium on C. crispus held in Halifax in 1972, McCandless reported that nuclear phase determined the type of carrageenan produced (46). McCandless et al in 1973 reported that the gametophyte of C. crispus produced mainly kappa carrageenan and a small but significant KCl soluble fraction very unlike lambda carrageenan, while the sporophyte produced lambda carrageenan almost exclusively (47). The same year Chen et al reported a higher kappa/lambda ratio in the gametophyte than in the sporophyte (12). The observation of a karyotype specificity of polysaccharide production presented the first example of isomorphic haploid/diploid algal generations producing chemically distinct major cell wall polysaccharides.

The introduction of immunological techniques as a tool useful in eludicating distinct carrageenan structures confirmed the genotypic control of polysaccharide production. Originally Johnston and McCandless (31) raised specific antibodies to "kappa" and "lambda" fractions (KCl insoluble and soluble fractions) in rabbits. Precipitin analysis showed antisera to be quite specific for the immunizing antigen i.e. very little cross-reactivity was observed. Fractions from different algal species were tested for antibody precipitability (32). Differences in precipitability were observed with carrageenan fractions prepared

similarly from different species. Cytologically, the use of fluorescein labelled anti-kappa carrageenan antibodies by Gordon and McCandless (27) had shown interaction of such antibodies with cell walls of gametophytic tissue. Once the genotypic specificity of carrageenan production was known, the immunological approach could be further refined and more specific antisera prepared. Gordon-Mills and McCandless found specific staining of sporophytic tissue with anti-lambda and of gametophytic tissue with anti-kappa antisera. Once fertilization has taken place and the diploid condition exists, anti-kappa carrageenan antibodies no longer bind specifically but anti-lambda carrageenan antibodies do bind (28). Similarly in the sporophyte, once sporogenesis had taken place (presumably with a reduction division), anti-kappa carrageenan antiserum showed reactivity with the tissue (28). This cytological work thus confirmed the ploidy control of polysaccharide synthesis and strongly suggested a "switch" mechanism effective within a very short time span.

Hosford and McCandless demonstrated in vitro that anti-kappa carrageenan antiserum raised in goats precipitated carrageenans from the gametophytes but not the tetrasporic phases of several algal species in the family Gigartinaceae but not outside this family (31). The gametophyte KCl soluble carrageenan showed lower precipitation of anti-kappa carrageenan antiserum unless this carrageenan fraction had been modified by alkaline borohydride treatment (31). Alkaline borohydride treatment causes elimination of C6 sulphate with the introduction of the 3,6-anhydro ring in appropriate sugar residues . (The reaction can take place whether or not there is a C2 sulphate present (68).) Iota carrageenan and furcellaran also precipitated anti-kappa

carrageenan antiserum as did alkaline borohydride treated lambda carrageenan. Since the presence of 3,6-anhydrogalactose was the known common denominator in polysaccharides giving precipitation, the authors proposed that it was involved in a determinant site. Although it proved to be more difficult to raise specific anti-lambda carrageenan antisera in goats, the specificity of certain antibody populations was demonstrated by selective absorption of antisera with heterologous antigens (24).

This in vitro and cytological work depends, as had previous studies, upon characterization of carrageenans, whether extracted or within the plant from a population of plants at a particular time point. In order to approach the problem of the synthesis of carrageenans and the regulation of such synthesis a time course of study of comparable plants was necessary. To approach this complex problem it was essential to know the optimal conditions for carrageenan synthesis.

As mentioned above many studies have been made of C. crispus in its natural habitat to determine the factors affecting carrageenan production. The most studied aspect was the seasonal variation in "kappa"/"lambda" ratio. Rigney (72) reported a drop in percentage of carrageenan in C. crispus plants from October to February in certain areas. Other locations showed a similar drop in August. The percentage of kappa carrageenan seemed to rise from June to October in some locations, while in others it seemed to remain stable. The size and degree of branching also affected carrageenan content and the "kappa"/"lambda" ratio. Thus location and season were considered to be

important factors. These variations can be explained now on the basis of different numbers of sporophytes and gametophytes in different locations and at different seasons. Chen and McLachlan (11) confirmed the presence of gametophytic plants with cystocarps throughout the year but noted that they were more abundant during the summer and autumn. Tetrasporic plants were also found throughout the year but were more common in winter and spring. Originally, within the framework of "seasonal variation" several more defined factors such as temperature, salinity, photoperiod, and/or intertidal exposure were invoked to explain variation (7, 9, 26). Certain conditions for maximal carrageenan production were suggested. (9, 26).

Perhaps the most productive approach was the use of in vitro culturing of C. crispus. As mentioned previously, the entire life cycle has been demonstrated in vitro (11). Prior to this report by Chen and McLachlan, Mathieson and Burns had investigated the correlation between photosynthesis and respiration and the light intensity, temperature, salinity, and dessication (44). Although the optimum light intensity for photosynthesis (as measured by oxygen evolution) was found to be about 1000 ft-c, the light intensity for maximum growth levelled off at 440 ft-c (see below). The optimum temperature for maximal apparent photosynthesis was 20 C and this was also the temperature showing the best photosynthesis/respiration ratio. C. crispus was found to be quite osmotolerant with salinities from 8^{o}/oo to 32^{o}/oo having relatively little effect on apparent photosynthesis although 24^{o}/oo was the optimal salinity. Dessication by exposure of plants to air, as might be expected with intertidal plants, had a great effect after a critical length of time (over 30 min).

In 1973 Burns and Mathieson (9) studied the effects of light, temperature, and salinity on carpospore germination and sporeling development (the latter measured by cell number). C. crispus showed an increase in growth with increasing light up to about 440 ft-c at which point a plateau was reached. Growth also increased with increasing temperature from 3 to 19 C. The results of temperature and salinity studies indicated an optimum for germination of 11 C and 35°/oo, while growth was greatest at 19 C and 40°/oo (although salinities between 25 and 45°/oo were tolerated (9).

Chen and McLachlan (11) also investigated the effects of temperature and light intensity and duration on spore germination and growth. They found no apparent differences in germination efficiency of carpospores incubated from 5 to 20 C under light intensities of 100 to 1000 ft-c and photoperiods of 8:16 (5 C), 12:12 (10 C), 10:14 (13 C), 12:12 (15 C), 16:18 (15 C), and 18:6 (20 C) light:dark. Sporeling development was most rapid at 15 C and 16:8 hr at about 500 ft-c. The 16 hr light period and 15 C temperature were essential for the production of sexually mature plants although large thalli were formed under all conditions. Conditions for maximum vegetative growth were the same for sporophytic and gametophytic plants. Chen and McLachlan also saw regeneration from "decapitated" apical frond regions, with the type of regeneration being dependent upon the extent of decapitation (11).

Neish et al used large tanks to study nutrient requirements and carrageenan production in C. crispus (53). They found that plants (tethered or freely circulating) in flowing seawater attained growth rates closely related to

temperature with the maximum rate at the highest temperature (20 C) - the same maximum observed by Mathieson and Burns (44). Neish et al found iron ineffective as a growth stimulant while phosphate and fixed nitrogen sources were effective. (Nutrients were added dropwise twice weekly: 5mM N; 0.5 nM PO_4^{2-}; and 0.1 nM iron; or in solid plaster of Paris cubes which dissolved to maintain an average concentration of 6×10^{-5} M nitrogen. Urea was found to be a poor nitrogen source compared to ammonium or nitrate compounds.

On the basis of observed rapid growth rate and lesser apparent susceptibility to epiphytes, plant T_4 was selected as parent of a clone for further studies (52). It was found that a high density of plants in culture tanks resulted in drastically reduced growth rates as might be expected as a result of nutrient and light deprivation. It was found that with recirculating seawater conditions "depleted" plants tend to bleach. This effect was counteracted by addition of nitrogenous fertilizers. When additional phosphate was not added fragmentation of plants was enhanced. The authors stated that N- enriched plants were higher in nitrogen and moisture but lower in dry matter and carrageenan while well nurtured plants in N-depleted seawater (temperature and light conditions being favourable) showed bleaching but the plants had higher dry matter and polysaccharide contents.

Salinity studies revealed that optimal growth rates were obtained with $31^{o}/oo$, the normal salinity of the area (52). These authors found, as had previous workers (9), that C. crispus was quite osmotolerant. They found that growth was equally rapid in tanks receiving Ca- or NH_4NO_3 as in tanks receiving

1/5 as much nitrogen (diammonium hydrogen phosphate) and concluded that nitrogen had been added in excess of requirements. Chen et al (11) found that different nitrogen sources: NH_4NO_3, $Ca(NO_3)_2$, and $(NH_4)_2SO_4$ were equally effective in promoting growth.

The logical progression from single sampling and in vitro life cycle observations would be to combine these two approaches so as to characterize the growth process and differentiation in pursuit of the chemical reaction involved. Use of radiotracers might be one very useful tool. Before the implication of genetic control of carrageenan synthesis was appreciated, Loewus et al (39) did a preliminary study with C. crispus, measuring incorporation of $^{35}SO_4^{2-}$ into KCl soluble and insoluble fractions of hot water extracts from unsorted plants. A level of 10^{-4}M SO_4^{2-} proved to be optimal for uptake of counts from the medium. (This same level has been reported to be optimal in other algal systems using similar procedures (23)). In the KCl soluble fraction 25% of the label was found to be dialysable. The "kappa" polysaccharide was eluted from Sephadex G-100 as a single broad band just after the void volume (the latter measured with Blue Dextran 2000). Alkaline borohydride modification of this fraction sharpened the peak but did not shift it. The "lambda" component showed a broader peak starting just after the void volume. Alkaline borohydride treatment of this fraction showed more label in higher molecular weight components. (The skewing of anthrone-reactive material toward lower molecular weight components before alkaline borohydride treatment was shifted more toward the labelling profile after treatment).

Earlier Bidwell and Ghosh (5) had undertaken labelling studies with

Fucus vesciculosus, a brown alga. They found that $^{35}SO_4^{2-}$ uptake was complex and not directly related to internal requirements. Light positively affected both uptake and incorporation into fucoidan. They also found that uptake was more directly related to "wounded" surface area rather than total surface area. Some exchange of sulphate with sulphated fucoidan was observed. The incorporation of $^{35}SO_4^{2-}$ into fucoidan was 20 to 600 times greater than that of ^{14}C (the ratio being dependent on the duration of the experiment). After 24 hrs the S/C ratio decreased slightly in the ethanol insoluble fucoidan while the S/C ratio in ethanol soluble fraction (containing inorganic sulphate) decreased dramatically because of a fourfold increase in ^{14}C with only a 10% increase in $^{35}SO_4^{2-}$.

Other early work investigated the uptake and incorporation of ^{14}C in marine algae. In 1963 Craigie studied the dark fixation of $^{14}CO_2$ by several multicellular marine algae (16) by measuring the uptake of $NaH^{14}CO_3$ from seawater. The brown algae tested removed more than the reds over a two hour period. In 1965 Majak et al studied photosynthesis in $^{14}CO_2$ over a two hour period with six Rhodophycean algal orders (41). Uptake values between 0.5 and 4.0 mg $hr^{-1}g^{-1}$ (fresh weight) were obtained. The macrophytes showed virtually no excretion of photosynthate, but the unicellular fresh-water red alga Porphyridium excreted 4.4% of such material in 2 hr. The three Gigartinales species studied were Ahnfeltia plicata, Cystoclonium purpureum, and Gigartina stellata. The latter, the most closely related to C. crispus of the algae tested, showed the lowest incorporation of ^{14}C. In the fractions tested G. stellata also showed the lowest accumulation of label in the chloroform soluble fraction of the evaporated alcohol extract, suggesting very low levels of label in lipid soluble

material. When water soluble compounds of this alcohol extract were subfractionated into cationic, anionic, and neutral fractions, G. stellata showed 88% of the label in the neutral fraction. Of this fraction 96% was in floridoside, a glycerol-galactose compound. Upon hydrolysis of alcohol insoluble residues, ^{14}C-glucose was the most prevalent component. Presumably this is because of hydrolysis of floridean starch.

More recent work on sulphate uptake and sulphate and ^{14}C incorporation has been carried out mainly in two algal systems - the unicellular red Porphyridium aerugineum by Ramus and coworkers (62 66) and in Fucus distichus by Quatrano and coworkers (30, 60, 61). The major findings with Fucus have been that sulphation of a non-sulphated fucan polymer takes place quite rapidly at a specific stage in development (60), and that a series of sulphated polymers exists (29).

In the Porphyridium system the unicellular alga normally has a capsular layer of sulphated polysaccharide (62). This polysaccharide, unlike carrageenans, is a heteropolymer of glucose, galactose, xylose, and other minor sugar components and had a sulphate content of 7.6% (62). Ramus first used ^{14}C pulse- chase experiments with $NaH^{14}CO_3$ to follow the excretion of this water soluble polysaccharide, separating the material into encapsulating material ("cell wall") and material solubilized in the medium. The uptake of ^{14}C became linear after a short lag period (about 15 min) and was light dependent. The ^{14}C labelled polysaccharide, isolated by cetyl pyridinium chloride (CPC) precipitation, was detected in the medium within 30 min after a pulse of 20 min. Ramus found cyclic changes in excretion with dark grown and alternate light-dark grown

cultures. He suggests that these changes are related more to rate of cell division rather than to photoperiod with the highest levels of excretion coinciding with the maximum rate of cell division. Ramus, using $^{35}SO_4^{2-}$, found sulphate uptake to be largely light dependent (65) and to be inhibited by molybdate (63). He also determined a K_m apparent for sulphate uptake. These uptake and incorporation studies with sulphate will be discussed more fully later.

Early attempts to label carrageenans in C. crispus using $NaH^{14}CO_3$ also showed cyclical variations with light conditions and with season of harvest (49). The incorporation reported was into carrageenans fractionated with KCl from unsorted plants and thus the seasonal "changes" are subject to the previously mentioned limitations of interpretation. Virtually all experiments showed increased incorporation in the light as might be expected for an energy dependent process in a photosynthetic organism. The "lambda" fraction showed the highest specific activity in all experiments (about 10-fold higher than "kappa" at 24 hr.). Fractionation of the "lambda" component on 1% agarose showed the highest molecular weight galactose polymers to be unlabelled but the labelled polysaccharide eluted from the column very closely behind the unlabelled fraction.

Knowing the significance of nuclear phase differences in carrageenan production, McCandless and Craigie used ^{14}C labelling with carposporic plants at different seasons (48). The specific activity of the KCl soluble fractions was always higher than the insoluble. In all but one experiment the specific activity of the alkali modified "μ" carrageenan was higher than that of the fraction

remaining soluble after alkaline borohydride. Seasonal differences were not statistically significant, however the rate of increase of specific activity was fastest in July and lowest in February experiments. The levels are compatible with the more advantageous summer growth conditions resulting in more active carrageenan synthesis.

The specificity of polysaccharide production thus has become an accepted fact, but the mechanism of this specific synthesis is totally unknown. Is the control at the level of transcription, translation, or is post translational regulation the dominant level of control processes regulating enzymes responsible for polysaccharide synthesis? In order to approach this complex array of possibilities, the primary requirement appeared to be knowledge of the actual pathway of biosynthesis. Despite extensive studies on carrageenan content, quality, and the factors regulating these characteristics, the fundamental knowledge about the path of synthesis and its regulation had not been extensively studied because of the complexities involved and the multitudinous factors influencing carrageenan synthesis. Even with the knowledge of life cycle stage control of synthesis, the copious problems associated with studying biochemical processes in an organism which grows approximately 8 cm per year obviated certain traditional approaches. Nevertheless the obvious necessity for knowledge of the biosynthetic pathway of carrageenan and the control thereof prompted the present work.

The first goal of this work was the development of suitable experimental conditions so that ^{35}S-sulphate and ^{14}C-bicarbonate labelling of carrageenan fractions to high specific activities would be possible. Once these

conditions were established it was possible to pursue the more specific goals which follow. Sulphate and bicarbonate uptake could be compared in sporophyte and gametophyte and the relationship, if any, between uptake and incorporation into carrageenan fractions could be studied. The life cycle stages also could be compared as to fractions labelled, amount and time sequence of labelling. In the gametophyte the effect of different strains, various conditions and effectors on labelling of carrageenan fractions could be studied. Ultimately it would be possible to confirm or rule out a precursor role for the gametophytic KCl soluble carrageenan fraction in kappa carrageenan synthesis and attempt to find the pathway of biosynthesis of kappa carrageenan.

MATERIALS AND METHODS

MATERIALS

I. CHONDRUS CRISPUS (STACKHOUSE)

Seaweed was kindly provided by Marine Colloids and Dr. J. Craigie. "Wild Type" C. crispus was harvested from Fink Cove, N. S. or the Northumberland Straits. The rapidly growing T_4 strain provided by Marine Colloids was from material cultured in enriched seawater at various densities. Two additional strains, Blue 7 and White 14, were also provided by Marine Colloids.

Seaweed was shipped by air on ice and maintained in an Aquarium Systems Inc. tank in Instant Ocean (Aquarium Systems Inc., Wickliffe, O. U.S.A.) at 12 C. In the second pulse-chase experiment thermostat failure caused temperature fluctuation (6 to 20 C) during the first week of maintenance.

II. ISOTOPES

$Na_2^{35}SO_4$ and $NaH^{14}CO_3$ were purchased from New England Nuclear (Boston, Mass., U.S.A.). The isotopes were diluted with unlabelled, analytical grade Na_2SO_4 and $NaHCO_3$ respectively to give the proper concentrations and required specific activities. Unless otherwise indicated radioactivity was added

at 1 μCi/ml medium.

III. CHEMICALS

All chemicals used were of analytical grade when available. Agarose (immunological grade) was purchased from Marine Colloids. Seawater for enriched seawater media was shipped by Dr. J. S. Craigie (Atlantic Regional Laboratory, National Research Council of Canada) from Nova Scotia by air freight. Prepared enzymes were purchased from Sigma Chemical Co. Kappa carrageenase was prepared from Pseudomonas carrageenovora (78) provided by Dr. W. Yaphe.

METHODS

I. CULTURE TECHNIQUES

1. Axenic Culture

Attempts were made to culture C. crispus axenically. The media used for these studies and subsequent studies are listed in Appendix A. C. crispus fronds were surface sterilized with a 1/5 dilution of Javex in seawater 2-15 min and/or in 80% ethanol for 1-3 min. Plants were then rinsed in sterile seawater 4 times and soaked in sterile seawater containing 0.01N HCl for 10 min. Plants were again rinsed in sterile seawater 4 times. Fronds were then segmented with a sterile scalpel. Small segments (about 1mm) were transferred to agar media

directly or crushed aseptically on a glass slide and then transferred to agar or liquid media. Larger segments were also tested (about 10mm) for viability on agar and in liquid media. Plant cultures were incubated in a growth chamber (Conviron, Controlled Environments Inc.) at 17C, 280-500 ft-c or in darkness (cultures covered in aluminum foil), at 80% relative humidity. Various light regimens were tested from continuous light to 16:8 hr: light; dark. Different parts of the algal tissue were tested i.e., apices, sub-apices, and stipes. Plant segments cultured on agar were transferred aseptically at 10 day intervals. Liquid cultures were transferred at 1, 2, or 3 week intervals. In order to help prevent contamination of agar plates they were wrapped in Saran wrap.

2. Protoplast preparation

For protoplast preparation two enzyme mixtures buffered to pH 7.0 were tried (adapted from higher plant methods) (Table 1).. Whole plants were kept in the dark for 2 days prior to treatment (14). The plant surface was sterilized with 70% ethanol for 2 min, then 1/5 Javex for 15 min. Plants were then immersed in 0.01N HCl in sterile seawater for 1 min (after being rinsed 3 times in sterile seawater). After final rinsing in sterile seawater, the plants were incubated for 3.5 hr in enzyme solutions. Cultures were then filtered through 2 layers of muslin into sterile centrifuge tubes. The filtrate was centrifuged at 100xg for 3 min. (14). Supernatant was aspirated and the precipitate was re-suspended in 10 ml 1B5(s) medium and recentrifuged. Supernatant was again aspirated and the precipitate resuspended in 1 ml medium. The suspension was pipetted into Petri plates in droplets and incubated in the growth chamber. Microscopic examination of the samples was carried out to

Table 1. Enzyme Solutions for Protoplast Preparations

	Compound	Volume of stock per 10 ml	Stock Concentration
Solution A.	Mannitol	0.5 ml	110 mg/ml
	$CaCl_2$	0.067 ml	B5 stock (Appen. A)
	Kappa carra-geenase	5ml	0.5 units/ml
	Cellulase *	10 mg	-----
	Pronase	1.0 ml	1 mg/ml
Solution B.	Sorbitol	0.5 ml	200 mg/ml
	$CaCl_2$	0.067 ml	B5 stock (Appendix A)
	Kappa carra-geenase	5 ml	0.5 units/ml
	Cellulase **	10 mg	-----
	Pronase	1.0 ml	1 mg/ml

* Cellulase (dry powder) was added directly to enzyme solutions.

determine effectiveness of the treatment.

3. "Clean" culture techniques

Low viability of axenic cultures and the problem of fungal and bacterial overgrowth led to the decision to use "clean" cultures. Whole plants were identified as to life cycle stage by visual identification of sori. Plants were desoried, scrubbed with a toothbrush in Instant Ocean, and cut into 20 mm segments (11 mm in the preliminary comparison of haploid and diploid plants). The segments were rinsed in sterile Instant Ocean and retransferred aseptically into sterile medium. Cultures were maintained in SWM3 medium (50) as described by Mayo-Harding (45). Cultures were incubated in the growth chamber at 17 C, 16:8 hr; light; dark, 280-400 ft-c (fluorescent and incandescent lighting) on a reciprocal shaker.

II. LABELLING STUDIES

1. Long term labelling

For long term labelling studies plants were rinsed in sterile Instant Ocean, after scrubbing in non-sterile Instant Ocean, and transferred aseptically to the artificial seawater medium of Novotny and Forman (56) containing radiolabel. Cultures were incubated in the growth chamber under the conditions given above unless otherwise indicated for specific experiments. The level of

$NaHCO_3$ was constant (2.36 mM) for all experiments. Sulphate concentrations are indicated for each experiment. Sterile radioisotope solutions were mixed with sterile cold carrier to give the proper final concentrations and specific activities when these stocks were mixed aseptically with the appropriate total volume of medium. Medium was then dispensed into individual flasks by sterile syringe. Effectors were added as sterile solutions to individual flasks where indicated. ^{35}S-sulphate uptake from the medium at the termination of the experiments was normally 20 - 25% or less of available label. At time of harvest medium was decanted and plant segments were rinsed in Instant Ocean on sintered glass filters and then air dried.

2. Pulse-chase studies

In pulse-chase experiments cultures were set up initially as described above. At the end of the pulse period medium was decanted and the plants were rinsed in Instant Ocean. Fresh medium of the same composition as the labelling medium but lacking radiolabel was used for the chase period. Harvesting was carried out as described above.

III. CARRAGEENAN EXTRACTION

Carrageenans were extracted by methods modified from those previously reported (47). Four methods were used with the particular method used being dependent upon the amount of algal material available, the timing of the experiment, and the necessity of specific activity determination.

1. Method I

Method I was essentially that reported previously (47). Air dried

material frozen in liquid nitrogen was milled in a ball mill. The powder was extracted with acetone, hot 80% ethanol, 95% ethanol, and diethyl ether and dried overnight in vacuo. Portions (0.2 - 0.4 g) were extracted with 0.5M $NaHCO_3$ at 90 C with vigorous stirring. The liquid was collected by pressure filtration through a celite pad and glass wool batt on top of a 12 μ Sartorious filter. Two re-extractions of the celite, glass wool and residue were done. Combined filtrates were filtered through 3.0 and 1.2 μ Sartorious filters. Carrageenans were precipitated with Cetavlon (cetyltrimethyl ammonium bromide). The Cetavlon was removed by washing the complex twice with distilled water and six times with saturated sodium acetate in ethanol. Whole carrageenan was precipitated with 2.5 volumes of ethanol and dried as above. Fractionation was achieved by resuspension in 0.3M KCl. Fractions were precipitated with 2.5 volumes of 2-propanol (NaCl to 1% concentration was added prior to precipitation), washed with 80% and absolute 2-propanol, and diethyl ether and dried as above.

The following modifications were made: a) the amount of material was reduced considerably; b) Celite was not used in pressure filtration; c) fractionation was achieved by addition of 0.6M KCl dropwise with stirring to a finai concentration of 0.3 M; d) KCl soluble and insoluble carrageenan fractions were separated by centrifugation at 20,000xg in a Sorvall centrifuge for 15 min.

2. Method II

Method II considerably shortened the time of extraction but excluded specific activity determinations. In this instance the KCl fractionation of whole

carrageenan was carried out as in Method I. The fractions were precipitated with Cetavlon (cetyl trimethyl ammonium bromide) added to a final concentration of 0.2% as in I. This Cetavlon precipitate however was then washed with 80% ethanol, 95% ethanol, and ethyl ether and dried overnight in vacuo over P_2O_5. The dried Cetavlon precipitates were then counted as carrageenan samples.

3. Method III

To shorten the extraction procedure and yet retain the option of specific activity determination, the following method was developed. (Table 2). After $NaHCO_3$ extraction, solid KCl was added to the final concentration of 0.3M to the filtrate, with stirring. After separation of soluble and insoluble carrageenan fractions by centrifugation, CPC (cetyl pyridinium chloride) was added to the KCl soluble and the resuspended KCl insoluble fractions to a final concentration of 0.2%. An aliquot was removed from the soluble fraction prior to CPC precipitation and KCl was added to a final concentration of 3M.

The CPC precipitates were collected by centrifugation and decomplexed by stirring (approximately 4 hr for KCl insoluble and 16 hr for KCl soluble fractions) in nearly saturated sodium acetate in 80% ethanol (after standing overnight in sodium acetate in ethanol, decantation, and resuspension). Carrageenan fractions obtained were washed and dried as previously reported (47).

4. Method IV

To test the effect of several effectors or conditions on carrageenan

Table 2 Carrageenan Extraction - Method III

Freeze air dried material in liquid nitrogen and mill 2 min. in ball mill. Extract with acetone for 1 hr, filter through glass fibre paper. Re-extract residue with boiling 80% ethanol.

Acetone-ethanol soluble

Dry residue in vacuo over P_2O_5

Extract in 0.5M $NaHCO_3$ for 2 hr at 85 - 90 C.

Filter hot through 12 and 3μ Sartorius filters

Filtrate

$NaHCO_3$ residue

Add solid KCl to 0.3M final concentration. Let stand overnight and collect precipitate by centrifugation.

Supernatant

Precipitate

Suspend in glass distilled water.

Add 2% QAC[1] to 0.2% final concentration
Collect precipitate by centrifugation.

KCl Soluble: QAC precipitate [2]

KCl Insoluble QAC precipitate [2]

Suspend in nearly saturated sodium acetate in 80% ethanol and stir for several hours. Collect precipitate on a sintered glass filter (preweighed). Wash with 95% ethanol and ethyl ether and dry overnight in vacuo over P_2O_5.

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[1] QAC = quaternary ammonium compound (either Cetavlon or CPC)

[2] In Method II these precipitates were collected on sintered glass filters, washed with 80 and 95% ethanol and ethyl ether and dried in vacuo over P_2O_5.

synthesis, a very short extraction procedure was developed for very small quantities of plant material. After harvesting and rinsing, plant material was cut into very small pieces (1-3 mm) and air dried as usual. The air dried samples were then extracted in 0.5M $NaHCO_3$ for 2 hr as in previous methods. Filtration was through 4 layers of cheesecloth rather than by pressure filtration through Sartorious filters. Solid KCl was added to the filtrates to a final concentration of 0.3M. Samples were treated subsequently as in Method III.

IV. ANALYTICAL METHODS

1. Sulphate determination

The following simple method for sulphate quantitation, based on Dodgson's turbidometric method, (20, 21) was developed to overcome the many problems associated with previously reported sulphate assays.

To 1.1 ml of sample containing 0-80 µg inorganic sulphate, 1.2 ml of 8% TCA was added, followed by 0.6 ml of agarose reagent (0.5 $BaCl_2$ in 100 ml of 0.01% agarose - left to stand overnight). After mixing and standing for 35 min, samples were shaken and the optical density was read at 500 nm in a spectrophotometer (versus a distilled water blank). A standard curve with 0, 30, 50, and 80 µg SO_4^{2-} was run in duplicate with unknown samples.

The effect of the presence of several common compounds on sulphate determination by this method was assessed during development of the method.

These data and data on linearity and reproducibility etc are given in Results I.

2. Protein determination

Protein quantitation was carried out by the method of Lowry et al (40), by determining optical density at 280 nm using a BSA (bovine serum albumen) standard curve, or by adaptation of the above sulphate method. The last procedure allowed rapid protein estimation over a wide range of concentrations. In this method the $BaCl_2$ was omitted from the agarose reagent. Protein samples from 25-2500 μg (BSA equivalents) can be determined (the standard curve is constructed in the estimated range of unknown samples with at least 3 concentrations being tested each time in duplicate). As in the sulphate assay the optical density was read at 500 nm after 35 to 40 min against a distilled water blank. The linearity and reproducibility of the method are reported in the Results. The method may be used in tandem with the sulphate assay to determine one compound in the presence of the other compound.

3. Carrageenan hydrolysis

The most common method for polysaccharide hydrolysis is heating the polysaccharide in acid solution in sealed tubes for various lengths of time. This method requires proper glass blowing equipment and expertise. Thus a simple method, based upon Butcher and Lowry's method for protein hydrolysis (10) was developed.

Polysaccharide samples in acid solution (1N HCl) were placed in tubes (the tube size varied according to sample volume), and an aliquot of light mineral oil was layered over the sample. A beaker was inverted over the tubes to exclude condensation. Samples were then hydrolysed in an autoclave at 15 psi for 4-5 hr. After cooling and gentle agitation to resuspend precipitated material, an aliquot was removed from the aqueous layer. Since high concentrations of acid affect the sulphate analysis (see Results I. 1.3) samples were diluted so that the final concentration of HCl was less than 0.1N (a level which does not affect the assay). Removal of an exact aliquot of the sample avoids the problem of transfer of contents from hydrolysis tubes.

Carbohydrate determination

Loewus' modification of the anthrone method (38) was used for quantitative carbohydrate analysis. Content of 3.6-anhydrogalactose was determined by the resorcinol method of Yaphe and Arsenault (83). The Somogyi-Nelson method (54) for reducing sugar content was used to determine kappa carrageenase activity.

5. Infrared analysis

Films for infrared analysis were prepared by a modified version of previously reported techniques (47). A 3 mg sample was placed on an AgCl disc. A few drops of boiling, glass distilled water were added and a uniform film was made by spreading the sample with a porcelain spatula covered with Parafilm

(42). Films were dried at 40 C for at least 1 hr, and were scanned in a Perkin-Elmer IR 283 spectrophotometer.

6. Chromatography

Descending paper chromatography on Whatman # 1 filter paper or ascending thin layer chromatography on cellulose were carried out in the following solvent systems: a) butanol: ethanol: water: :1:1:1 (79) b) butanol: ethanol: water: 4:1:1: plus 3% CPC (79). Paper chromatography was carried out for 3.5 to 5.5 hr while TLC was for 1.5 to 2.5 hr.

Sugar, polysaccharide sulphates, and anhydrogalactose were detected by the alkaline silver nitrate (77)., toluidine blue (51), and Seliwanoff (81) reagents respectively. Standards included galactose, galactose-6-phosphate, glucose, and glucose-6-sulphate.

7. Determination of mean and variation from the mean

Most samples were run in duplicate and thus the variation from the mean is not a standard deviation (S.D.) but arithmetic difference from the mean showing the range of values obtained. In most cases when more than two samples were used, the range of values was expressed as a + and - from the mean and the sample number is given. This designation makes skewing of data more obvious

than simple maximal and minimal data values.

In the reproducibility experiment and in a few other cases the S.D. was calculated and in these cases values are expressed as mean $\bar{X} \pm$ S.D. For such calculations and determination of best fit straight lines, a multi-function pre-programmed SR 9190 R calculator (Commodore Business Machines Inc., Stockton on Tees, England) was used.

RESULTS

I. ANALYTICAL METHODS

1. Sulphate determination

1.1 Fig. 3 shows standard curves for sulphate determination using Na_2SO_4, K_2SO_4, and $(NH_4)_2SO_4$. Two agarose concentrations were tested with Na_2SO_4. At the higher agarose concentration (0.02%) the assay lost linearity at a lower sulphate concentration. It is evident that the cations tested had no effect on sulphate determination in the assay.

The turbidity is quite stable after 30 to 35 min:

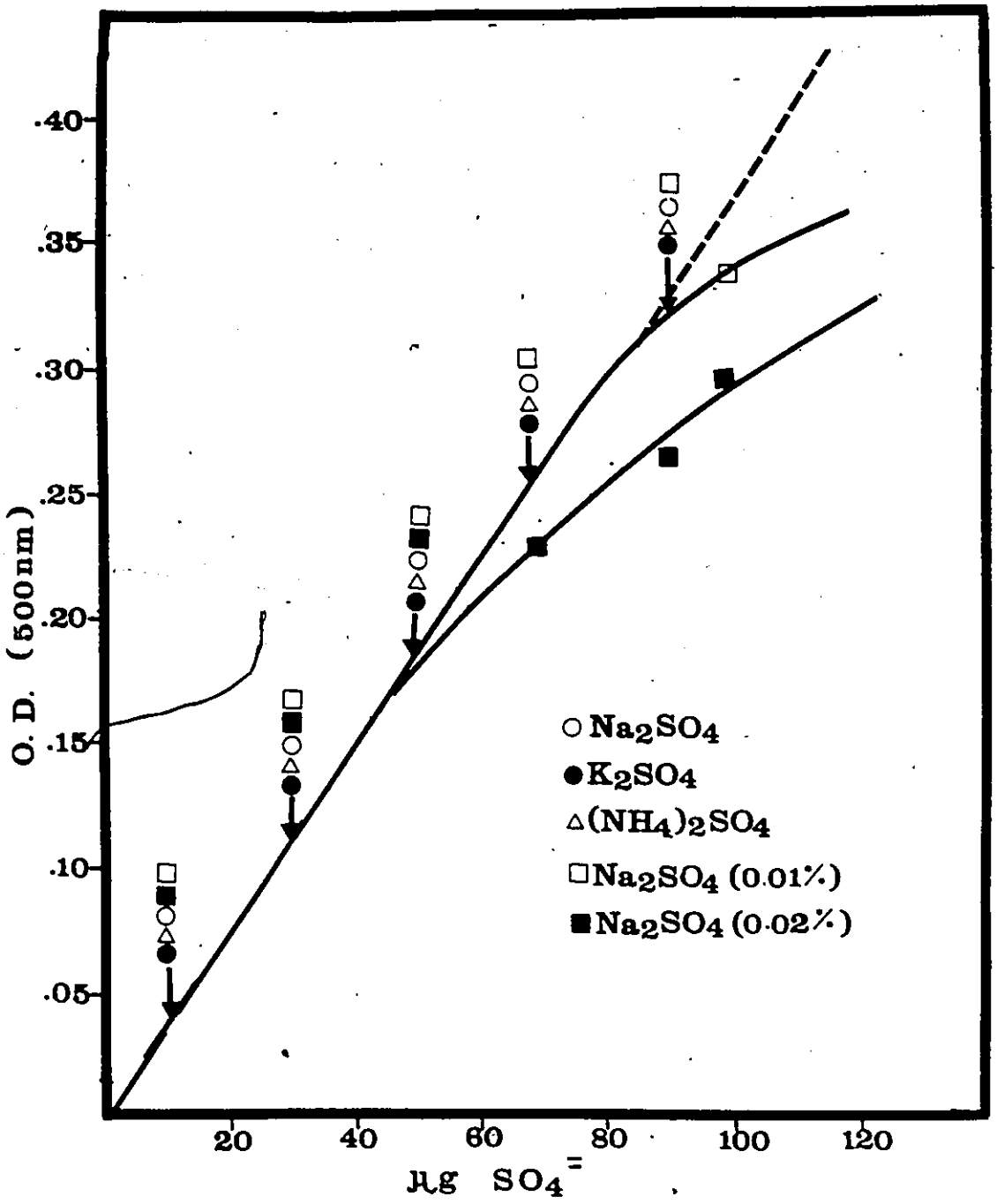
Table a * Development of Turbidity with Time

	Time (min)				
	10	20	30	35	56
O.D.$_{500\ nm}$	.129	.185	.187	.186	.184
(50 µg)	.174	.176	.181	.180	.180

* Brief tables in text are designated with lower case letters.

The assay is highly reproducible. Not only the slopes of standard curves but also actual optical density readings were very similar on different days. For example, on three different days (usingthe same reagent) mean optical density readings ($\pm$ standard deviation) $\bar{X}$ = .197$\pm$.033, .311$\pm$.006, and .387$\pm$.019 were obtained for 0, 30 and 50 µg ($\bar{X}$ is the mean of six values). Thus the

Fig. 3 Optical density at 500 nm versus sulphate concentration using Na_2SO_4 ○, K_2SO_4 ● , and $(NH_4)\ SO_4$ ▲ with 0.01% agarose reagent. With Na_2SO_4 as standard the assays were repeated on another day with 0.01% □ or 0.02% ■ agarose.


.40
.35
.30
.25
.20
.15
.10
.05
O. D. (500nm)
20
40
60
80
100
120
µg SO4=
○ Na2SO4
● K2SO4
△ (NH4)2SO4
□ Na2SO4 (0.01%)
■ Na2SO4 (0.02%)

maximum variation over these three days was less than 5% for a given concentration. Even with different reagents, standard curve values on different days (over 5 mo apart) were similar as shown below. The reagent is stable but loses sensitivity after about three weeks (linearity maintained but slope decreased).

Table b Optical Density Values on Different Days with the Same or Different Reagents

	O.D.$_{500\ nm}$					
$\mu g\ SO_4^{2-}$	7/3/78		22/9/77		27/10/77	
0	.192	0*	.195	0*	.198	0*
30	.309	.117	.309	.114	.296	.098
50	.389	.197	.377	.182	.352	.154
80	.478	.286	.448	.253	.429	.231

* Values corrected for blank

1.2 In order to compare this turbidometric assay with another assay, that of Jones and Letham (33), sulphate was determined in the hydrolysates of a number of carrageenan samples. These data are given in Table 3. The ratio column indicates that the methods agree closely in quantitative determination of inorganic sulphate.

1.3 Several common reagents, many of which interfere with other sulphate assays, were tested with the turbidometric assay. A level of 50 $\mu g\ SO_4^{2-}$ was used in all assays. The results are summarized in Table 4. Although protein interferes with the sulphate assay as expected, it is possible to use the turbidometric protein assay (see below) in tandem in order to determine sulphate in the presence of protein.

Table 3 · A Comparison of the Turbidometric and Jones and Letham Methods for Determination of SO_4^{2-} in Carrageenan Hydrolysates

Carrageenan	µg SO_4^{2-}/mg carrageenan		Ratio**
	Turbidometric	Jones and Letham	
1.	143	115	0.81
2.	214	190	0.89
3.	127	113	0.89
4.	217	207	0.95
5.	99.7	93.2	0.93
6.	201	171	0.85
7.	110	100	0.91
8.	220	204	0.93
9.	261	250	0.96
10.	310	311	1.00
11.	303	302	1.00
12.	303	305	1.00
13.	297	299	1.00
14.	340	284	0.83
15.	290	266	0.92
16.	272	265	0.97

17.	313	285	0.91
18.	295	278	0.94
19.	307	270	0.88
20.	256	231	0.90
21.	236	200	0.85
22.	191	158	0.83
23.	423	372	0.88
78.	284	309	1.10
79.	296	277	0.94
80.	379	371	0.98
81.	337	306	0.91
82.	368	350	0.95
83.	365	353	0.97
84.	323	293	0.91
85.	292	280	0.96
86.	186	175	0.94
87.	216	190	0.88
88.	159	154	0.97
89.	233	216	0.93

* All samples were run in duplicate.

** Ratio = $\frac{\mu g\ SO_4/mg \text{ polysaccharide by Jones and Letham}}{\mu g\ SO_4/mg \text{ polysaccharide by turbidometric}}$

Table 4. The Effect of Various Compounds on the Turbidometric Sulphate Assay.**

	Concentration (in sample)	ΔO.D.$_{500\ nm}$
1) Interference by:		
Kappa carrageenan	0.9 mg/ml	-0.022
Ethanol	43%	-0.025
Tris-HCl buffer, pH 7	0.5 M	-0.028
Tris-acetate buffer, pH 7	0.5 M	-0.036
Tris-acetate buffer, pH 7	0.5 M	-0.036
HCl	0.3 N	-0.021
	0.6 N	-0.047
	1.0 N	-0.125
NaOH	0.6 N	-0.123
HNO_3	1.0 N	-0.344
Cetavlon *	0.2%	+1.486
BSA	0.9 mg/ml	+0.524

2) No Interference by:

HCl	0.1 N	+0.001
NaOH	0.1 N	+0.005
	0.3 N	-0.010
D-galactose	0.9 mg/ml	0
D-galactose-6-phosphate	0.9 mg/ml	0
D-glucose	0.9 mg/ml	-0.005
D-glucose-6-sulphate	0.9 mg/ml	0
$MgCl_2$	0.114 M	-0.004
NaH_2PO_4	0.114 M	+01.011

* Cetyl trimethyl ammonium bromide

** All samples were run in duplicate.

Table c Use of Sulphate and Protein Assays in Tandem

Sample	$O.D_{500\ nm}$ minus blank* Agarose - $BaCl_2$	Agarose
250 µg protein +50 µg SO_4^{2-}	0.288	0.135
250 µg protein		0.140
50 µg SO_4^{2-}	0.159	0.000

* Samples were run in duplicate

2. Protein Determination

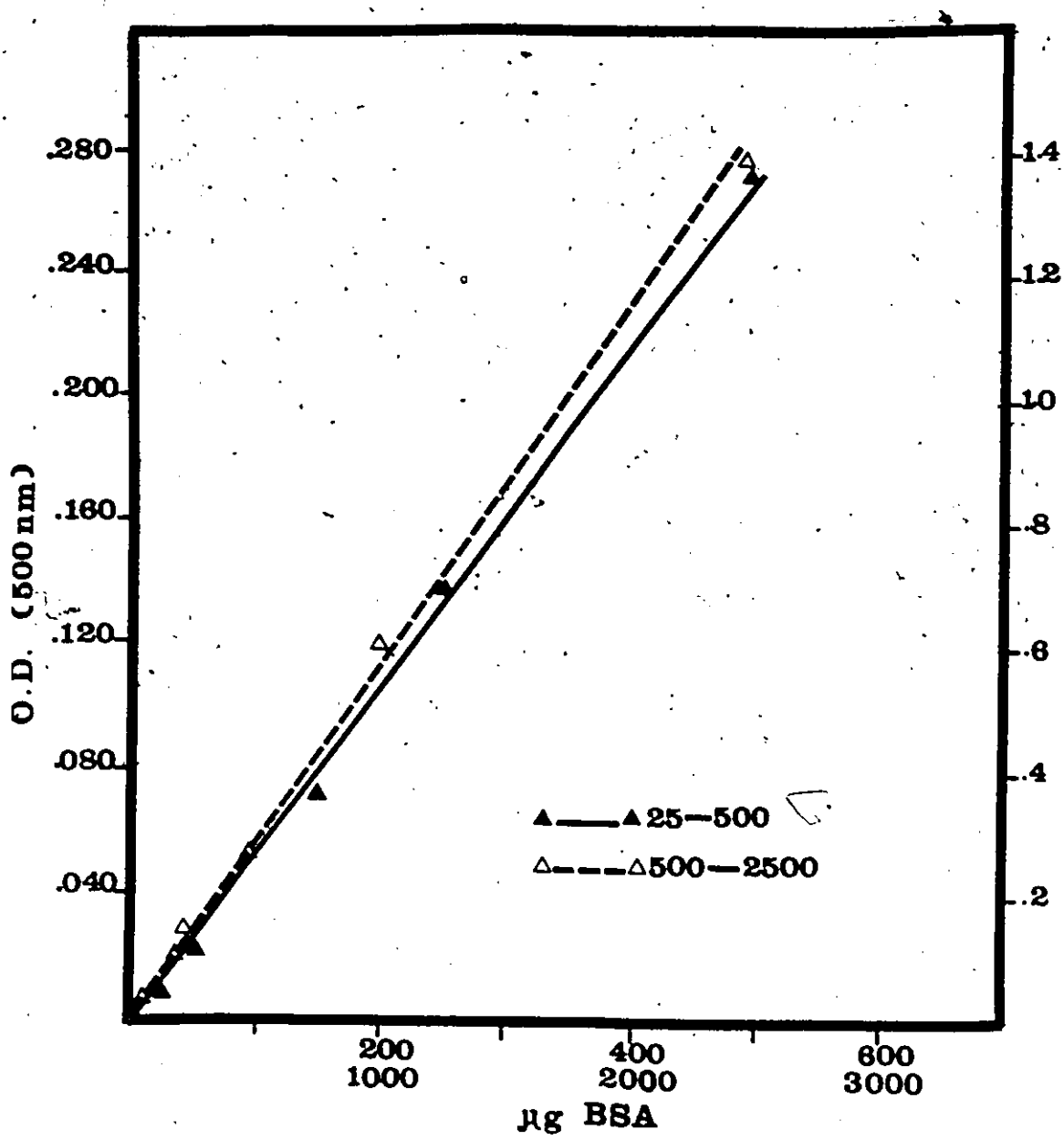
2.1 The turbidometric sulphate assay was adapted to protein determination simply by the exclusion of $BaCl_2$ from the agarose reagent. Fig. 4 shows that the assay is linear over the range of 25 to 2500 µg protein. The turbidity is quite stable after 35 to 40 min although it continues to increase slightly (0.001 O.D. unit per 6 min (see below)). Linearity is achieved after 35 min.

Table d Development of Turbidity in Protein Assay

	Time (min)						
	5	18	30	37	44	60	93
$O.D._{500\ nm}$	0.105	0.116	0.124	0.127	0.131	0.136	0.149
(250 µg BSA)	0.107	0.118	0.126	0.131	0.135	0.142	0.155

Like the sulphate assay, the protein assay is consistent. Using 100µg

Fig. 4 Optical density at 500 nm versus the protein concentration using the turbidometric assay. The double scale used (25-500 and 500-2500 μg) illustrates linearity over the hundred-fold range without unnecessary scale expansion.


.280
.240
.200
.160
.120
.080
.040
O.D. (500 nm)
1.4
1.2
1.0
.8
.6
.4
.2
▲——▲25—500
△- - -△500—2500
200
1000
400
2000
600
3000
µg BSA

bovine serum albumen a mean optical density ($\pm$ standard deviation) $\bar{X}=0.0692^{+}$ 0.0011 was obtained. (This represents a concentration range of $\bar{X}$ =99.2 $\pm$ 1.8 µg. $\bar{X}$ is the mean of 5 determinations.

2.2 The turbidometric assay was compared with another protein assay, that of Lowry et al (40). The results are shown in Table 5. As with other methods the turbidometric assay gives different optical density readings with similar amounts of different proteins. Nevertheless, other proteins do show a linear relationship between concentration and $O.D._{500\ nm}$ over a wide concentration range as evidenced by pronase ($O.D._{500\ nm}$= 0.021, 0.045, 0.068, 0.099, and 0.129 for 110, 220, 330, 440, and 550 µg respectively). As expected proteins such as gelatin and glycoproteins such as mucin which are not precipitable by TCA cannot be determined by the turbidometric assay.

2.3 Several common substances were tested for effect on the turbidometric assay (250 µg bovine serum albumen). Many compounds which interfere with other proteins assays did not affect this assay (Table 6). It is especially noteworthy that the amino acids tested and mercaptoethanol did not interfere.

II. CULTURING

1. Axenic cultures and protoplast preparation

Although some signs of apical swelling possibly indicating growth

Table 5. Comparison of the Turbidometric and Lowry et al Methods of Protein Determination **

Protein	mg Bovine Serum Albumen equivalents per mg protein		
	Lowry et al	Turbidometric	Ratio *
Bovine serum albumen	1.000	1.000	1.00
Deoxyribonuclease	0.920	0.908	1.04
Inorganic pyrophosphatase	0.906	0.239	3.79
[illegible]onase	0.524	0.321	0.63
Gelatin	0.580	0	
Mucin	0.461	0	

$$*\text{Ratio} = \frac{\text{mg bovine serum albumen equivalents by Lowry et al method}}{\text{mg bovine serum albumen equivalents by Turbidometric method}}$$

** Duplicate samples

Table 6. The Effect of Various Compounds on the Turbidometric Protein Assay

	Concentration (in sample)	ΔO.D.$_{500\ nm}$
1) Interfering Compounds		
$NaPO_4$ buffer, pH 7.7	0.1M	-0.015
Kappa carrageenan	1.4 mg/ml	-0.117
KCl insoluble carrageenan	455 µg/ml	-0.035
	45.5 µg/ml	-0.020
Tris-acetate buffer, pH 7.0	0.55 M	+0.042
HCl	0.55 N	+0.030
Cetyl pyridinium chloride	1%	+2.072
2) Non-interfering Compounds		
Casamino acids	0.9 mg/ml	-0.008
Glycine	0.9 mg/ml	-0.007
Tyrosine	0.4 mg	-0.001
Phosphate buffered saline, pH 7	0.01 M	-0.007
$CaCl_2$	0.9 mg/ml	-0.003
Tris-HCL buffer, pH 7	0.55 M	-0.003
Mercaptoethanol	0.10 M	+0.003

D-galactose	0.9 mg/ml	+0.002
D-galactose-6-phosphate	0.9 mg/ml	+0.002
D-glucose	0.9 mg/ml	+0.004
D-glucose-6-sulphate	0.9 mg/ml	-0.002
$MgCl_2$	0.10 M	-0.005
NaOH	0.55 N	-0.001
KCl	0.33 M	-0.001
Na_2EDTA	0.11 M	-0.008

appeared in most axenic gametophyte and sporophyte cultures (liquid and agar cultures), the plants were extremely slow in showing such signs (usually 10 days to 3 weeks). Also the problems of bacterial and fungal contamination proved to be insurmountable. The combination of these factors made further pursuit of this line of research impractical.

An attempt was made to prepare protoplasts from gametophyte material as a possible way to obtain cultures which would synthesize cell wall and matrix polysaccharides rapidly. "Protoplasts" were prepared as described (Methods I.2.). Microscopic examination of such C. crispus "protoplast preparations" showed both enzyme treatments to be very effective in producing "protoplast-like" entities and/or small clumps of cells. Samples were stained with toluidine blue to detect the presence of sulphated polysaccharides. Clumps of cells showed evidence of some characteristic metachromatic staining but many single "cells" were negative to such staining indicating the absence of significant amounts of sulphated polysaccharide. Segments treated with the second enzyme solution (using sorbitol as osmotic agent) showed a lower incidence of cell clumps in the sample examined.

The "protoplast-like" cultures failed to proliferate noticeably however in either of the media tested (Appendix A). After 10 days of culture most plates were contaminated with fungus.

2. "Clean" cultures

Using the SWM3 medium (50) as adapted by Harding (45) (SWM-4) both

gametophyte and sporophyte "clean" segments (Methods I.3.) were cultivated successfully. Visible apical growth was generally evident within a week. After 2 to 3 weeks in culture both gametophyte and sporophyte cultures rapidly synthesized carrageenans as shown with ^{35}S and ^{14}C labelling studies.

When attempts were made to culture T_4 gametophyte segments in enriched seawater medium (Appendix A), the algae bleached rapidly and fragmented. At high light conditions (400-500 ft-c) initial signs of bleaching appeared within 24 hr. At lower light levels (200-250 ft-c) bleaching was delayed but was severe within 6 days. Attempts to prevent or reverse bleaching by addition of fixed nitrogen sources were unsuccessful (Table 7).

Gametophyte plants maintained in Instant Ocean under the same conditions did not show bleaching. Similarly plants incubated in the artificial seawater medium of Novotny and Forman (56) for one week without sulphate and a further 2 days in this medium with 0.1 mM sulphate did not bleach or fragment.

As shown in Figs. 5 and 6 carrageenans show characteristic peaks in infrared spectra (68, 75). The 3,6 anhydrogalactose peak is between 930 and 940 cm^{-1} (935 in the KCl soluble and 928-932 cm^{-1} in the insoluble (Figs. 5 and 6)). All sulphated polysaccharides exhibit a broad absorption band at 1230-1250 cm^{-1}. When sulphate is present on the C2 of the 3,6 anhydro galactose unit there is an absorption band at 805-810 cm^{-1} which is proportional to 3,6 anhydro galactose-2-sulphate content (1). A C4 sulphate on the 3-linked unit causes an absorption in the 840-850 cm^{-1} region (840 for the soluble and 845 cm^{-1} for the insoluble

Table 7. The Effect of Nitrogen Supplementation of Enriched Seawater on T_4 Cultures.

Culture	Nitrogen[a] Source	Frequency of Addition of N	Observations[b]
1. B[c]	-	-	Bleached, Fragmented
2. N[d]	-	-	Bleached, Intact
3. B	NH_4NO_3	Daily	Bleached, Fragmented
4. B	$NaNO_3$	Daily	Bleached, Fragmented
5. N	NH_4NO_3	Daily	Bleached, Intact
6. N	$NaNO_3$	Daily	Bleached, Intact
7. B	NH_4NO_3	Alternate days	Bleached, Fragmented
8. B	$NaNO_3$	Alternate days	Bleached, Fragmented
9. N	NH_4NO_3	Alternate days	Bleached, Intact
10. N	$NaNO_3$	Alternate days	Bleached, Intact

[a] NH_4NO_3 0.03 ml of 1M stock

$NaNO_3$ 0.06 ml of 1M stock

[b] Observations made 3 days later

[c] B= "bleached" cultures previously grown in unsupplemented medium

[d] N= "new" cultures maintained in Instant Ocean

Conditions: Light 500 ft-c, 16: 8h:: light:dark; 17 C;SWM-4 medium.

Fig. 5 Infrared spectra of 0.3M KCl insoluble (kappa) carrageenans from cultured and non-cultured gametophytes. From top to bottom they are non-cultured wild type, cultured wild type, and T_4.

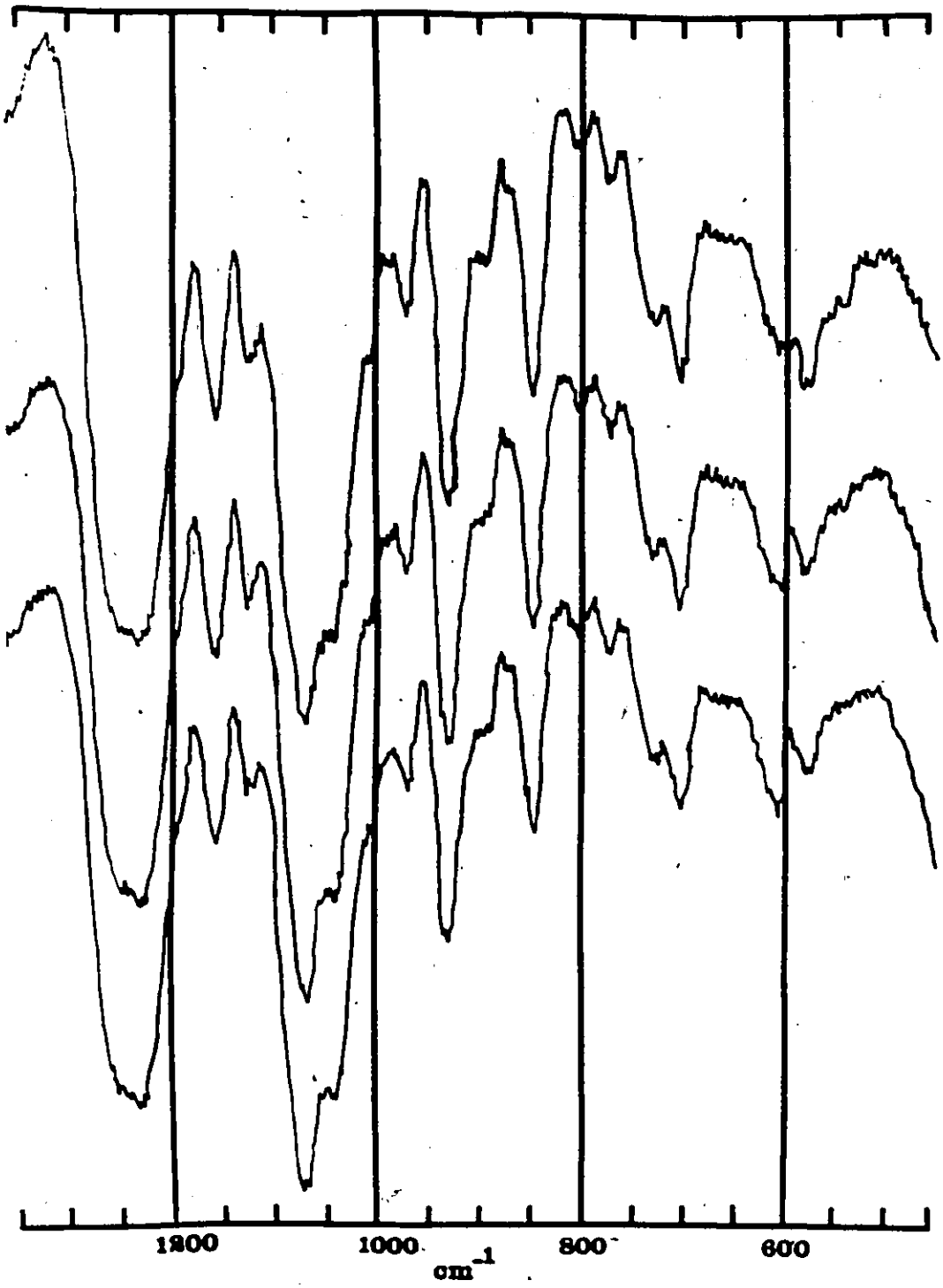
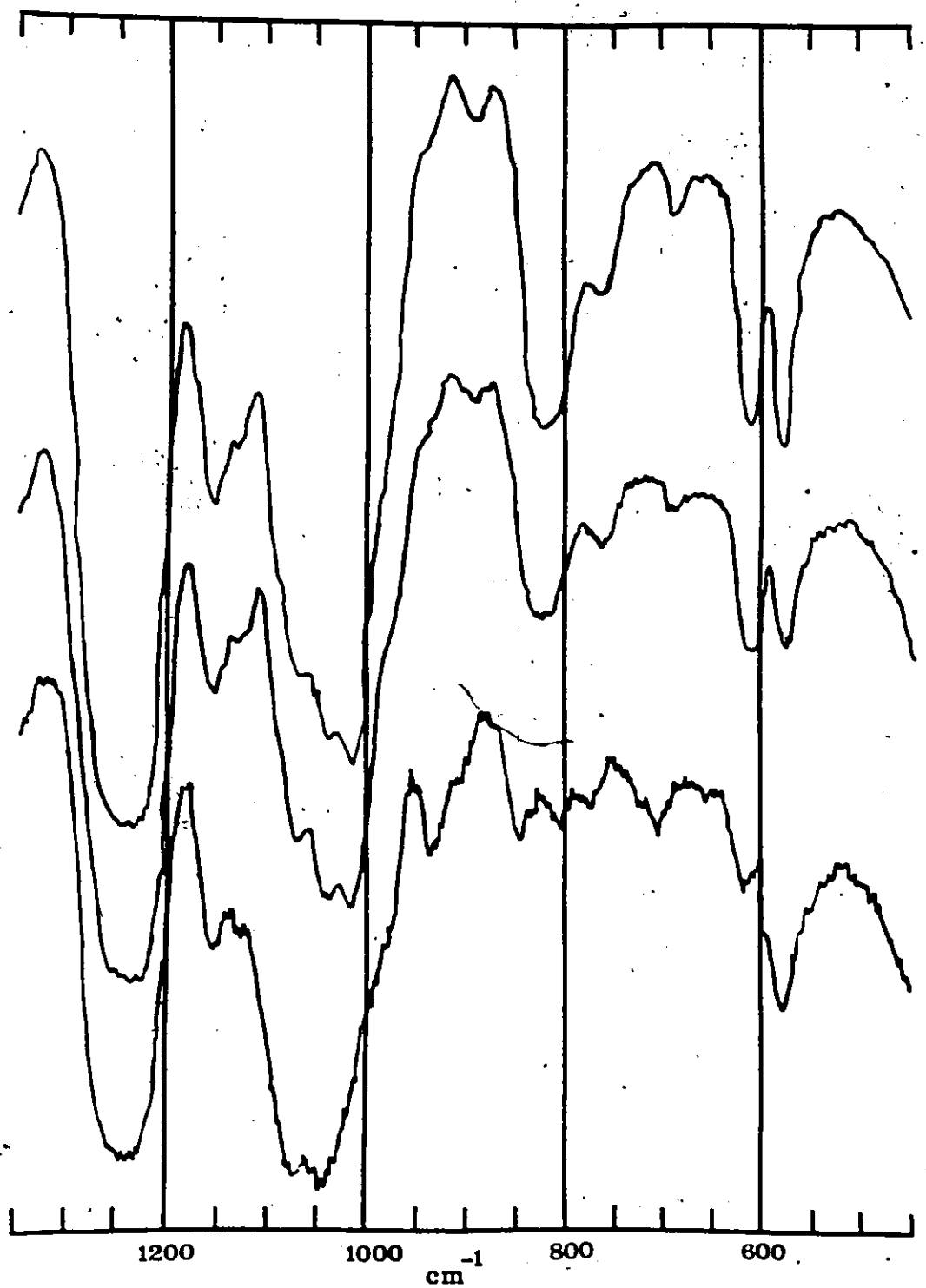

1200
1000
800
600
cm-1

Fig. 6 Infrared spectra of 0.3M KCl soluble carrageenans from non-cultured sporophyte (top); cultured sporophyte (middle); and T_4 gametophyte (bottom).


1200
1000
800
600
cm -1

fraction (45) and Figs. 5 and 6). The absorption peak for C2 sulphates not in a 3,6 anhydro galactose unit occurs at about 830 cm^{-1} (75). A primary equatorial sulphate such as a C6 sulphate in the 4-linked galactose unit absorbs in the 820-830 cm^{-1} range (75). The broad band at 1030-1050 cm^{-1} represents axial and equatorial hydroxyl groups (75).

Although culturing resulted in enhanced kappa carrageenen synthesis (Table 10), the polysaccharides produced were typical in the cultured material and also in T_4 gametophyte (Fig. 5). Similarly, cultured sporophytes produced typical lambda carrageenans (Fig. 6.). The infrared spectrum from T_4 gametophyte of 0.3M KCl soluble carrageenan is included to illustrate the difference between gametophytic and sporophytic 0.3M KCl soluble carrageenans.

III. LABELLING STUDIES

1. "Clean" cultures with prolonged labelling

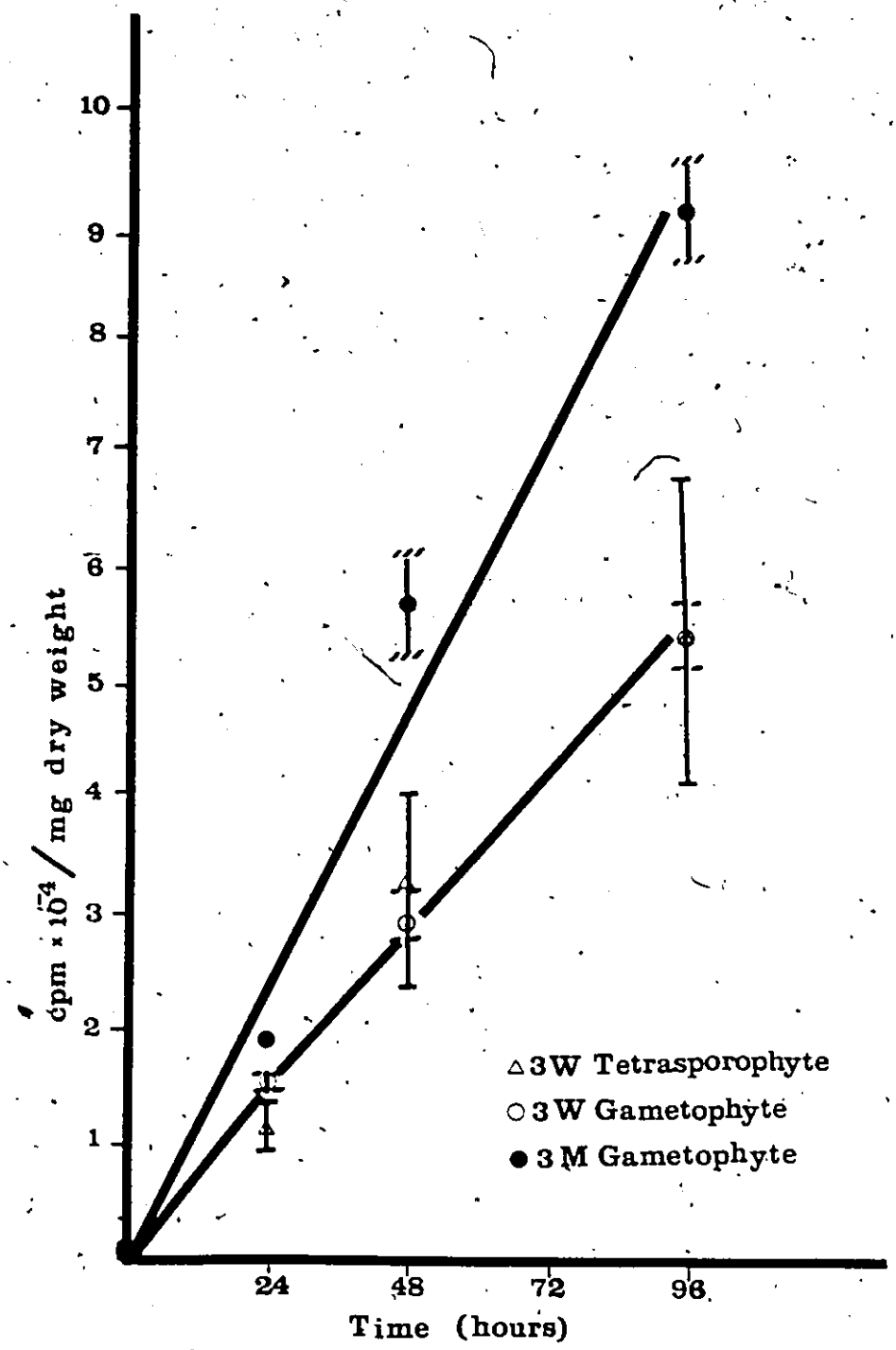
1.1 Sulphate uptake *

* Uptake values in 1.1 represent uptake from medium containing 0.1 mM ^{35}S sulphate at a specific activity of 1 μ Ci/ml medium.

(i) Uptake by gametophyte compared with sporophyte

The preliminary experiment with cultured gametophytic and sporophytic tissue showed little difference in sulphate uptake by segments cultured for three weeks (3W cultures) (Fig. 7). The gametophyte tissues cultured for three months (3M gametophyte) did show a somewhat higher uptake however. Similarly haploid and diploid plants from a different harvest site

Fig. 7 Uptake of $^{35}SO_4^{2-}$ from the medium by 3W tetrasporic Δ, 3W gametophytic ○ and 3M gametophytic ● plants. Error bars are represented for the cultures by solid black, wide stripe, and narrow strip bars, respectively.


10
9
8
7
6
5
4
3
2
1
cpm × 10⁴ / mg dry weight
24
48
72
96
Time (hours)
△ 3W Tetrasporophyte
○ 3W Gametophyte
● 3M Gametophyte

cultured for a similar time (24 - 39 days) had rates of uptake similar to each other but different from the 3W cultures of the previous experiment.

Table e Sulphate Uptake by Gametophyte and Sporophyte Cultured 24-39 days (cpm x 10^{-4}/mg dry weight)

	24 hr[a]	48 hr[b]
Gametophyte	$4.68^{+4.67}_{-1.69}$	7.49 ± 2.07
Sporophyte	$4.53^{+1.62}_{-1.28}$	9.65 ± 0.38

a = mean of 4 values $\pm$ variation range from the mean

b = mean of 2 values $\pm$ variation from the mean

Thus it appears that factors other than karytope may determine sulphate uptake.

Gametophytic material from yet another harvest showed uptake rates intermediate between the values from the two above mentioned experiments after two weeks of <u>in vitro</u> culturing.

Table f Sulphate Uptake by Gametophyte Cultured 2 weeks (cpm x 10^{-4}/mg dry weight

	24 hr[a]	48 hr[a]	72 hr[b]	96 hr[b]
2W Gametophyte	2.72	4.97	5.98	7.49
	$^{+0.51}_{-0.74}$	$^{+0.95}_{-1.20}$	± 0.92	± 0.82

a = mean of 6 values $\pm$ variation from the mean

b = mean of 2 values $\pm$ variation from the mean

In a preliminary experiment (not using duplicate samples) a comparison was made between cultured gametophytic material, non-cultured gametophytic material, and non-cultured T_4 gametophytic material under

different light conditions. In this case the cultured material was maintained under low light conditions (150 ft-c) until 24 hr before T_o. The results are shown in Table 8. Over the first 48 hr it would appear that T_4 has a lower uptake of sulphate regardless of light. By 96 hr the non-cultured gametophyte seems to have the highest uptake. Also the non-cultured material seems to have an equivalent or lower uptake under high light conditions as opposed to that of the cultured material or the rapidly growing T_4 gametophyte.

(ii) Uptake by T_4

In experiments such as the above it is crucial to know the reproducibility involved. The reproducibility of such experiments was estimated using a statistically significant number of samples at each time point. The results obtained using T_4 gametophyte are given below.

Table g Sulphate Uptake by T_4 Gametophyte (cpm x 10^{-4}/mg dry weight)

Time	2 hr	24 hr	48 hr	72 hr	96 hr
Sample Number	10	10	10	5	5
$\bar{X}$	0.320	0.975	1.58	2.99	4.87
$\pm$ S. D.	$\pm$ 0.26	$\pm$ 0.28	$\pm$ 0.29	$\pm$ 0.44	$\pm$ 0.73
% variation from the mean	81.0	28.7	18.4	14.7	15.0

Table 8. Uptake of $^{35}SO_4^{2-}$ by Cultured and Non-cultured Gametophytic C. crispus (cpm x 10^{-4}/mg dry weight) *

Type of Gametophyte		Cultured		Non-cultured		T_4	
Light conditions		High	Low	High	Low	High	Low
Uptake at:	24 hr	3.15	2.81	2.82	2.71	2.18	1.18
	48 hr	3.39	3.06	3.24	3.51	2.55	2.06
	72 hr	3.84	3.54	4.17	5.19	3.90	3.15
	96 hr	4.56	4.11	5.55	6.45	4.92	4.11

* Single samples

Conditions: High Light 400 ft-c, low light 150 ft-c; 16: 8h:: light: dark; 17 C, $Na_2{}^{35}SO_4$ (0.1 mM) 1 µCi/ml medium.

As might be expected the variation is greatest at shorter time periods because of the smaller number of counts involved.

1.2 Sulphate Incorporation

(i) Gametophyte compared with Sporophyte

A preliminary experiment was undertaken to determine what levels of ^{35}S incorporation could be achieved using cultured gametophytic and sporophytic material. Because of the small amount of tetrasporic material available, duplicates were pooled for all samples for carrageenan extraction after acetone extraction. Two types of gametophyte cultures were used, one type cultured for three weeks as were the sporophytic plants and the other from three month cultures. The latter plant segments showed the morphological characteristics of rapidly growing plants (53) i.e. bleaching, many small finger-like apical projections. The distribution of ^{35}S in various fractions is shown in Table 9A. Acetone - ethanol fractions contained similar levels of ^{35}S in all samples (with perhaps a slightly higher level in the 3M gametophyte). Total incorporation into total carrageenan (soluble and insoluble fractions) differed greatly in different karyotypes. The incorporation showed distinctly ploidy related patterns as would be anticipated from chemical analysis (47). Relatively low levels of ^{35}S remained in the $NaHCO_3$ extracted residue; the higher counts in the case of the 3M gametophyte may be a result of higher incorporation of sulphur into protein during the labelling period.

The rather unexpected differences in ^{35}S content in the Cetavlon supernatant suggested further investigation of the nature of the compound(s)

Table 9. Incorporation of ^{35}S in Gametophytic and Sporophytic C. crispus (preliminary comparison) *

A. Distribution of ^{35}S in Various Fractions (cpm x 10^{-4}/mg dry weight)

	3W Sporophyte		3W Gametophyte		3M Gametophyt
	48 hr	96 hr	48 hr	96 hr	96hr
Acetone-ethanol soluble	0.30	0.50	0.33	0.45	0.65
Carrageenans					
Total	0.93	1.9	0.98	0.84	1.4
KCl soluble	0.90	1.8	0.40	0.15	0.33
KCl insoluble	0.025	0.023	0.57	0.69	1.1
Residue	0.038	0.090	0.08	0.05	0.18
Cetavlon supernatant	0.047	0.031	0.56	0.62	0.041

B. Specific Activities of Carrageenans (cpm x 10^{-4}/mg galactose equivalent)

	3W Sporophyte		3W Gametophyte		3M Gametophyte
	48 hr	96 hr	48 hr	96 hr	96 hr
KCl soluble	10.3	11.3	14.1	18.7	45.9
KCl insoluble	5.55	7.50	3.66	4.34	11.0

* Duplicate samples pooled after acetone-ethanol extraction.

Conditions: Light 400 ft-c, 16: 8h:: light:dark, 17 C; plants precultured 3 weeks (3W) or 3 months (3M); $Na_2{}^{35}SO_4$ (0.1 mM) 1 μ Ci/ml medium.

containing this activity might be useful. Ten times as much ^{35}S was found in the 3W gametophyte as in other cultures.

The specific activities of the carrageenan fractions are shown in Table 9B. All values appeared relatively constant during the 48 to 96 hr period. Stability might be expected in the major fractions (soluble in the sporophyte and insoluble in the gametophyte) because of the large background of unlabelled carrageenan. The relatively constant specific activity of the KCl soluble fraction in the gametophyte suggests a steady state situation assuming no real change in amount of the fraction (the per cent yields on a dry weight basis support this assumption). Although the 3M gametophyte showed much higher specific activities than the 3W plants, the ratio of KCl soluble/KCl insoluble specific activities is almost identical. The life cycle stage influence on this ratio and manipulation of the ratio by modification of growth conditions will be discussed. Despite alteration of the ratio, however, the actual polysaccharides produced in the cultured samples showed characteristics of normal material. No shifts or deletions of characteristic peaks were observed in infrared spectra (see Fig 5 and 6). Actual yields of carrageenans based on dry weight (14.3^{+}_{-} 5.1%) or acetone-extracted dry weight (41.5^{+}_{-} 14.4%) were similar in both types of plants.

Another preliminary experiment (not using duplicates) was conducted in order to see if the time consuming culture techniques were in fact a significant factor in the high rate of incorporation. Two different light intensities were also tested as presumably light would be extremely important in regulating growth status of the algae. As well as normal gametophyte, cultured

and uncultured, the gametophytic "mutant" T_4,a rapidly growing strain (52), was tested. The results after 96 hr of labelling are shown in Table 10. The most striking observations are the high levels of ^{35}S in acetone - ethanol fractions in non-cultured material compared to other samples and the high level of incorporation into KCl insoluble carrageenan in the T_4 strain. In carrageenan fractions the order of incorporation was always T_4 > cultured > non-cultured gametophyte. Since higher light conditions appeared to favour incorporation into kappa carrageenan, the higher light level was used in virtually all subsequent experiments.

The gametophytic cultured material was further tested over the 96 hr labelling period for ^{35}S incorporation. All samples were labelled, extracted, and analyzed in duplicate. Pulse-chase samples were included in order to obtain some solid evidence as to the sequence of incorporation into various fractions. Results are shown in Table 11 . No great differences in distribution appeared except that it seemed that the 96 hr KCl soluble fraction contained fewer counts in both chased and non-chased samples. The specific activity of the KCl soluble fraction of the chased sample was much lower than the 48 hr labelled fraction while the specific activity of the 96 hr non-chased sample was similar to the 48 hr sample as shown in Table 11. This seems to confirm the dynamic nature of the KCl soluble fractions.

When more tetrasporic material became available, another comparative labelling experiment was conducted. The relative incorporation of ^{14}C was also measured (see Results III 1.4 i). The distribution of ^{35}S is shown in Table 12. The total incorporation of ^{35}S into the acetone-ethanol soluble

Table 10. The Effect of Light Intensity on Incorporation of ^{35}S into C. crispus Gametophytes *

A. Total Counts (cpm x 10^{-4}/mg dry weight)

	Cultured		Non-Cultured		T_4	
Light	High	Low	High	Low	High	Low
Acetone-ethanol soluble	1.05	0.963	2.74	3.59	0.643	0.494
KCl soluble carrageenan	0.284	0.359	0.207	0.156	0.334	0.586
KCl insoluble carrageenan	0.748	0.552	0.334	0.328	1.24	1.22

B. Specific Activities of Carrageenan Fractions

(cpm x 10^{-4}/mg galactose equivalent)

Light	Cultured		Non-cultured		T_4	
	High	Low	High	Low	High	Low
KCl soluble carrageenan	34.1	29.2	28.9	20.9	27.3	35.3
KCl insoluble carrageenan	3.0	2.2	1.2	1.5	5.5	5.6

* Single samples

Conditions: High Light 400 ft-c, low light 150 ft-c; 16: 8h:: light: dark; 17 C, $Na_2{}^{35}SO_4$ (0.1 mM) 1 µCi/ml medium.

Table 11. The Incorporation of ^{35}S by Cultured C. crispus Gametophyte

A. Distribution of Label in Various Fractions (cpm x 10^{-4}/mg dry weight)[1]

	48 hr	96 hr	96 hr_c[2]
Acetone-ethanol soluble	0.82±0.17	1.6±0.06	0.88±0.07
Carrageenans			
KCl soluble	0.61±0.28	0.19±0.09	0.12±0.12
KCl insoluble	1.01±0.37	1.32±0.22	0.84±0.09

B. Specific Activities of KCl Soluble and Insoluble Carrageenans (cpm x 10^{-4}/mg galactose equivalent)[1]

	48 hr	96 hr	96 hr_c[2]
KCl soluble	40.5±3.6	37.2±8.6	13.0±8.3
KCl insoluble	5.4±1.6	8.3±1.3	5.1±0.1

[1]Average values of duplicates are given and the range indicated.

Subscript c refers to plant samples "pulsed" for 48 hr and "chased" for 48 hr

[2](After 48 hr plants were removed from labelling medium, rinsed in Instant Ocean, ar resuspended in medium without radiolabel).

Conditions: Light 400 ft-c, 16:8h:: light:dark; 17C; plants precultured 2 weeks; $Na_2{}^{35}SO_4$ (0.1 mM) 1 μCi/ml medium.

Table 12. Distribution of ^{35}S in C. crispus Sporophyte and Gametophyte *

(second comparison)

A. Total Counts (cpm x 10^{-4}/mg dry weight)

	Gametophyte		Sporophyte	
	24 hr	48 hr	24 hr	48 hr
Acetone-ethanol soluble	0.807 ±0.073	2.10 ±0.48	0.949 ±0.12	2.33 ±0.12
Carrageenans				
KCl soluble	0.592 ±0.073	1.53 ±0.49	1.05 ±0.01	2.41 ±0.05
KCl insoluble	0.169 ±0.036	0.523 ±0.26	Negligible	

B. Specific Activities of Carageenan Fractions

(cpm X 10^{-4}/mg galactose equivalent)

	Gametophyte		Sporophytes	
	24 hr	48 hr	24 hr	48 hr
KCl soluble carrageenan	9.01 ±3.8	20.7 ±10.1	6.42 ±0.17	16.4 ±1.2
KCl insoluble carrageenan	1.01 ±0.09	3.22 ±0.95	- -	- -

* Duplicate samples

Conditions: Light 400 ft-c, 16: 8h:: light:dark; 17 C; plants precultured 24 -39 days; $Na_2{}^{35}SO_4$ (0.1 mM) 1 μCi/ml medium.

material increased in both types of plant tissue from 24 to 48 hr. Very similar levels occurred in sporophyte and gametophyte at a given time period.

As in the acetone-ethanol soluble fraction, incorporation into KCl soluble carrageenan approximately doubled in the diploid plant over the 24 to 48 hr period. A somewhat smaller increase in labelling was seen in the KCl soluble gametophytic carageenan.

The specific activity of the KCl soluble fraction of the sporophyte increased considerably from 24 to 48 hr while the specific activity of the gametophyte KCl soluble carrageenan fraction seemed to increase but showed great variability at 48 hr. The specific activity of the KCl insoluble carrageenan in the haploid plant increased over the labelling period despite the large "background" of unlabelled material. As with the preliminary experiment yields of total carrageenan were similar in gametophyte and sporophyte (25.9$\pm$4.3% of dry weight or 45.0$\pm$6.8% of acetone-extracted dry weight).

(ii) Sulphate incorporation in T_4

A major concern is the reproducibility of data. As stated in Materials and Methods, the extraction procedure is very time-consuming so for most experiments multiple samples were not used for each time point. An experiment was carried out therefore with a sufficient number of samples to determine reliability of results and yield information on standard deviation. Results of this experiment with 5 samples per time point are shown in Table 13 A & B. It is immediately evident that reproducibility is good and that there is good

Table 13. ^{35}S Labelling of T_4 Gametophyte (reproducibility experiment)*

A. Distribution of ^{35}S in T_4 (cpm x 10^{-2}/mg dry weight) $\bar{X}\pm$ Standard Deviation

Fraction	48 hr	96 hr
Acetone-ethanol soluble	45.4±8.3	148±8.9
KCl soluble carrageenan	8.43±2.38	40.9±9.12
KCl insoluble carrageenan	1.11±0.412	7.18±1.48

B. Specific Activity of Carrageenan Fractions (cpm x 10^{-4}/mg galactose)

	48 hr	96 hr
KCl soluble carrageenan	0.794±0.224	3.96±0.784
KCl insoluble carrageenan	0.0971±0.0387	0.783±0.291

*Five samples per time point.

Conditions: Light 250 ft-c, 16:8h:: light:dark; 17 C; $Na_2{}^{35}SO_4$ (0.1 mM) 1 µCi/ml medium.

separation of time points. Expressed as per cent variation, based on cpm x 10^{-2}/ mg dry weight, the values are 18.3, 28.2 and 37.1 at 48 hr and 6.0, 22.3 and 20.6 at 96 hr for acetone-ethanol soluble material, KCl soluble and KCl insoluble carrageenan respectively. The specific activity values also show good differences between time points. (Table 13B). The per cent variation in specific activity values are 28.2, and 39.9 at 48 hr and 19.8 and 37.2 at 96 hr for the KCl soluble and insoluble carrageenans respectively. One might have anticipated a greater variation in the KCl insoluble carrageenan fractions because of the large background of unlabelled material.

1.3 ^{14}C Uptake

The assessment of ^{14}C uptake from the medium when $NaHCO_3$ is used is complicated by rapid exchange with ambient CO_2. Also the contribution of CO_2 through plant respiration must be considered in determining actual uptake when media sampling is the method of measurement. However, conditions of the experiments reported here have been set to give a substantial net carbon fixation and it is this net uptake and incorporation that was measured. In most experiments the amount of passive CO_2 exchange was estimated by incubating media samples with no algae present under the same conditions as plant cultures. Corrections could thus be made for exchange and net uptake could be measured.

The size of the exchange problem over an extended labelling period is illustrated by the percentage of original label remaining. The amount of label

remaining at 24 hr was 26.3^{+}_{-} 1.53% and at 48 hr $7.83^{+2.12}_{-0.9}$ % of the original (o time) label (values represent the mean of 3 samples $^{+}_{-}$ variation range from the mean). By 96 hr less than 1% of the original label was present and thus only the first 48 hr has been reported.

Net uptake values varied with the seaweed sample tested as had ^{35}S uptake. Three different samples of T_4 gametophyte gave uptake values (cpm x 10^{-4}/mg dry weight) of $3.90^{+0.84}_{-1.11}$ (4), $3.43^{+0.31}_{-0.40}$ (4), at 24 hr and $1.13^{+0.33}_{-0.38}$ (4); $0.506^{+0.041}_{-0.069}$ (4), and $1.15^{+}_{-}0.03$ (3) at 48 hr (Values are means $^{+}_{-}$ range of variation; numbers in brackets represent the number of samples). Gametophyte uptake values were consistently slightly lower than those of the sporophyte samples but the differences were not large enough to be considered significant. Values for the sporophyte (cpm x 10^{-4}/mg dry weight) were $10.4^{+2.0}_{-1.5}$ (4) at 24 hr and 3.01^{+}_{-} 0.33 (2) at 48 hr. The gametophyte uptake values were $8.20^{+0.52}_{-0.35}$(4) at 24 hr and $2.4^{+}_{-}0.09$ (2) at 48 hr.

1.4 ^{14}C Incorporation

^{14}C incorporation in gametophyte and sporophyte

To approach the question of biosynthesis of carrageenans the order and time sequence of formation of the carbon skeleton must be known as well as the timing of sulphation. The use of ^{14}C should yield information regarding this problem. Supplying ^{14}C in inorganic form circumvents difficulties of uptake of organic compounds, reported to be minimal or nil in certain red algae (64). Unfortunately because of the similarity in energy of radiation of ^{14}C and ^{35}S

double labelling techniques cannot be applied. However, because of the good reproducibility, experiments done in tandem can yield data virtually as useful regarding sulphation and carbon skeleton synthesis.

Cultured gametophyte material was labelled over a 96 hr period. Pulse-chase samples were included as they had been in the ^{35}S labelling experiment (Table 11). Results given in Table 14 show similar total incorporation and specific activity values in chased and non-chased samples. The possible causes of this similarity will be discussed.

Both gametophytic and sporophytic cultures were then tested for ^{14}C incorporation between 24 and 48 hr. Table 15 shows that levels of ^{14}C in the acetone-ethanol fraction are stable over the 24 to 48 hr period and roughly equivalent in both types of plants. The sporophyte showed negligible counts in the very small KCl insoluble portion (plants were extracted by Method III).

At 24 hr the sporophyte and gametophyte showed similar incorporation into "KCl soluble carrageenans" although of course this represents synthesis of lambda carrageenan primarily in the sporophyte and "K precursor" in the gametophyte. By 48 hr, however, the sporophyte showed greater incorporation into the soluble fraction, while the gametophyte did not. The level of radioactivity in the KCl insoluble carrageenan in the gametophyte remained stationary over the 24 to 48 hr period. Specific activity values did not change significantly in specific carrageenan fractions in a given phase (Table 15B). Although total incorporation into "KCl soluble" carrageenans appeared higher in

Table 14. The Incorporation of ^{14}C by Cultured C. crispus Gametophyte

A. Distribution of Label in Various Fractions

(cpm x 10^{-4}/mg dry weight)[1]

	48 hr	96 hr	96 hr_c[2]
Acetone-ethanol soluble	10.7±0.3	7.0±1.8	10.2±0.9
Carrageenans			
KCl soluble	0.29±0[2]	0.35±0.04	0.4±0.01
KCl insoluble	0.76±0.03	1.08±0.04	0.85±0.01

B. Specific Activity of Carrageenan Fractions

(cpm x 10^{-4}/mg galactose)[1]

	48 hr	96 hr	96 hr_c
KCl soluble	19.3±4.6	20.5±10.1	25.8±6.0
KCl insoluble	4.7±0.5	5.8±0.7	6.0±1.1

[1] Average values of the duplicates are given with the range indicated.

[2] Subscript c refers to plant samples "pulsed for 48 hr and "chased" for 48 hr. (After 48 hr plants were removed from labelling medium, rinsed in Instant Ocean, and resuspended in medium without radiolabel).

Conditions: Light 400 ft-c, 16:8h:: light:dark; 17C; plants precultured 2 weeks; $NaH^{14}CO_3$ (2.36 mM) 1 µCi/ml medium.

Table 15. Distribution of ^{14}C in C. crispus Gametophyte and Sporophyte*

A. Total Incorporation (cpm x 10^{-4}/mg dry weight)

	Gametophyte		Sporophyte	
	24 hr	48 hr	24 hr	48 hr
Acetone -	12.6	12.8	15.9	15.9
ethanol soluble	±1.0	±0.2	±4.65	±1.4
Carrageenans				
KCl soluble	0.304	0.315	0.485	0.678
	±0.12	±0.013	±0.15	±0.06
KCl insoluble	0.126	0.161		
	±0.019	±0.026	Negligible	

B. Specific Activities of Carrageenan Fractions

(cpm x 10^{-4}/mg galactose)

	Gametophytes		Sporophytes	
	24 hr	48 hr	24 hr	48 hr
KCl soluble	7.38	6.95	3.45	4.56
Carrageenan	±0.79	±0.36	±1.2	±0.12
KCl insoluble	0.822	1.01	-	-
Carrageenans	±0.034	±0.23	-	-

* Duplicate samples

Conditions: Light 400 ft-c, 16:8h:: light;dark; 17 C; plants precultured 24 -39 days; $NaH^{14}CO_3$ (2.36 mM) 1 μCi/ml medium.

the sporophyte at 48 hr the specific activity of ^{14}C in galactose equivalents was similar.

2. Effect of Various Conditions on the Uptake and/or Incorporation of $^{35}SO_4^{2-}$ and ^{14}C

Because of previous reports of the effects of molybdate on sulphate uptake and sulphated polysaccharide synthesis and the effect of fixed nitrogen sources on the latter, these and other compounds were tested for effect on sulphate uptake and incorporation. Table 16 shows the effect of several compounds on sulphate uptake by T_4 gametophyte. Results are expressed as per cent of control. It is perhaps surprising that very little effect is demonstrated with any of the compounds tested.

The particular strain and/or physiological status of C. crispus was also found to affect sulphate uptake (Results III 1. ii, Table 16b). The Blue 7 strain was morphologically similar to the T_4 strain, while the White 16 strain was morphologically similar in frond thickness and size to the wild type and was sporulating at the time of testing. Uptake values for Blue 7 appeared somewhat elevated while White 16 showed somewhat lower uptake compared to the T_4 samples.

The level of sulphate in the medium had a profound effect on sulphate uptake in T_4 C. crispus as shown in Figs. 8 and 9. Concentrations over the range from 0.02 to 10.0 mM were tested (Trial 1: 0.02, 0.10, 1.0 and 10.0 mM and Trial 2: 0.02, 0.05, 0.10, 0.50, 1.0, 5.0 and 10.0 mM). The data implied a rapid initial uptake within the first hour followed by a slower but linear uptake over the next 4 hours at most concentrations tested. The rate curves in Figs. 8 and 9 show the

Table 16. Effect of Various Compounds and Algal Strain on Sulphate Uptake by C. crispus

A. Effect of Various Compounds (per cent of control) on T_4

	5 hr	6 hr	24 hr	48 hr
10^{-3}M Mo 0_4^{2-}*		166.1	106.0	141.0
		±114	± 35.7	± 21.3
10^{-4}M WO_4^{2-}*	96.4		143	104
	±32.6		±32.5	±23.1
$5x10^{-2}$M NO_3^-*		94.8	141	117
		±55.3	±72.1	±19.4
10^{-4}M NH_4Cl	80.1		105	127
	±12.8		±5.30	±10.5
10^{-4}M urea	67.1		167	117
	±10.8		±8.00	±10.0

* Duplicate samples. All controls were also run in duplicate.

B. Effect of Algal Strain (cpm x 10^{-4}/mg dry weight)

	2 hr	3.5 hr	5 hr
Strain: T_4	$0.891^{+0.26}_{-0.23}$ (6) *	$1.10^{+0.12}_{-0.11}$ (4)	$1.39^{+0.07}$ (2)
Blue 7	$1.33^{+}_{-}0.10$ (2)	$1,50^{+}_{-}0.01$ (2)	$1.56^{+}_{-}0.03$ (2)
White 16	$0.70^{+}_{-}0.08$ (2)	$0.72^{+}_{-}0.07$ (2)	0.89^{+}_{-}.-1 (2)

* Number in brackets refers to number of samples. Values refer to mean $^{+}_{-}$ variation from the mean.

Conditions: Light 320 ft-c, 16:8h:: light:dark; 17 C; $Na_2{}^{35}SO_4$ (0.1mM) 1 µCi/ml medium.

best fit straight lines determined using a pre-programmed SR 9190R calculator (see Materials and Methods).

Uptake rates calculated were:

Table h Sulphate Uptake By T_4 Gametophyte at Various Sulphate Levels

Sulphate Level (mM)	^{-35}S-sulphate Activity Added (cpm x 10^{-6}/µmol)		Uptake Rate (n mol hr^{-1} mg^{-1} dry weight)	
	Trial 1	Trial 2	Trial 1	Trial 2
0.02	62.8	161	0.02	0.14
0.05		63.7		0.034
0.10	12.7	32.3	0.16	0.12
0.50		6.62		0.50
1.00	1.96	3.37	0.98	0.74
5.00		0.664		3.90
10.0	0.271	0.335	5.32	3.80

When all data for a given trial were plotted in 1/V versus 1/S plots no meaningful kinetic constants could be determined. Similarly if the three lower concentrations in Trial 1 were plotted a negative V_{max} value was obtained. When the three higher concentrations were used (0.10, 1.0, and 10 mM), however,

Fig. 8 Uptake rate curves for $^{35}SO_4^{2-}$ at 0.02, 0.10, 1.00, and 10.0 mM sulphate (Trial 1). Circles represent duplicate values and squares represent best fit points.

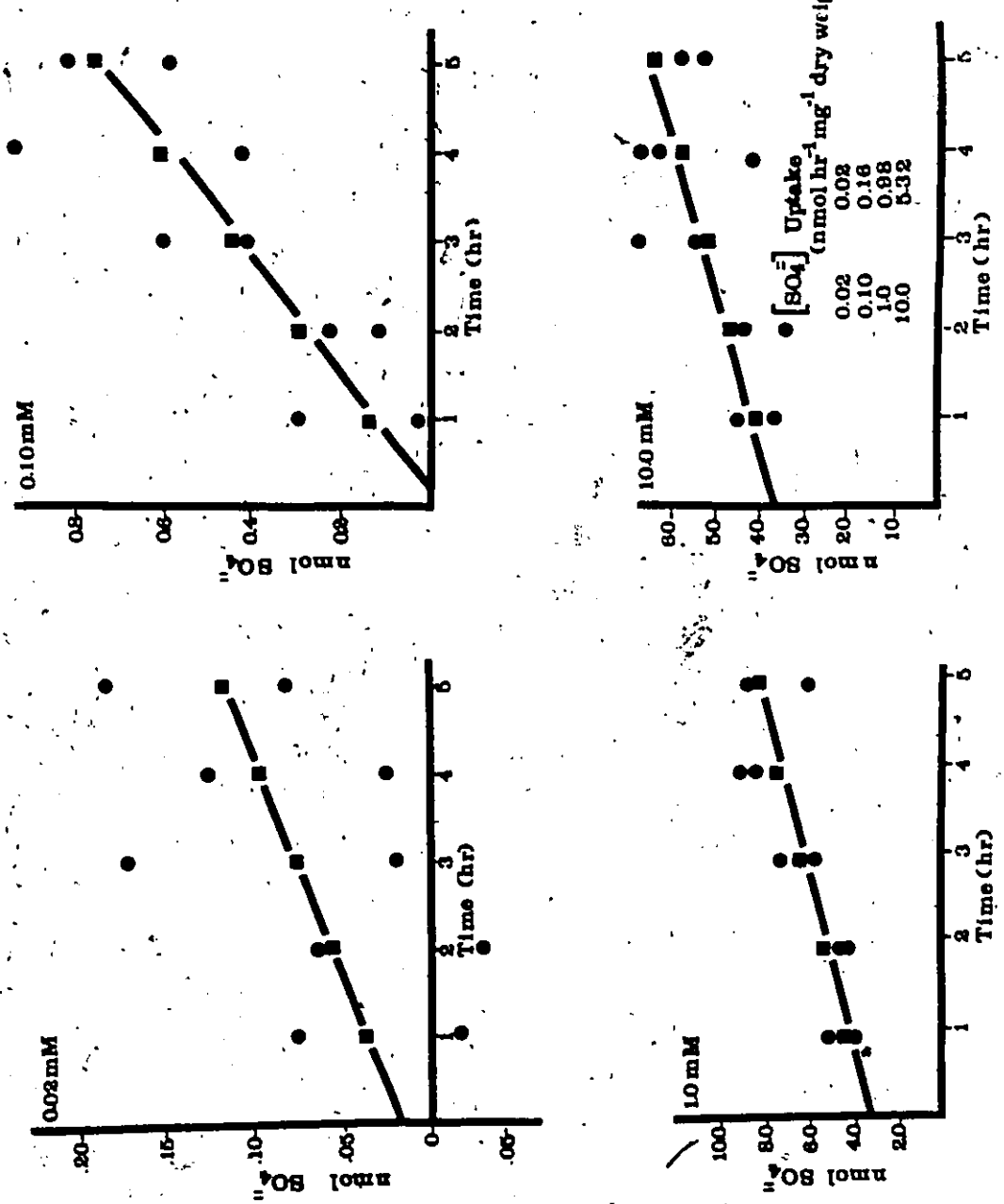
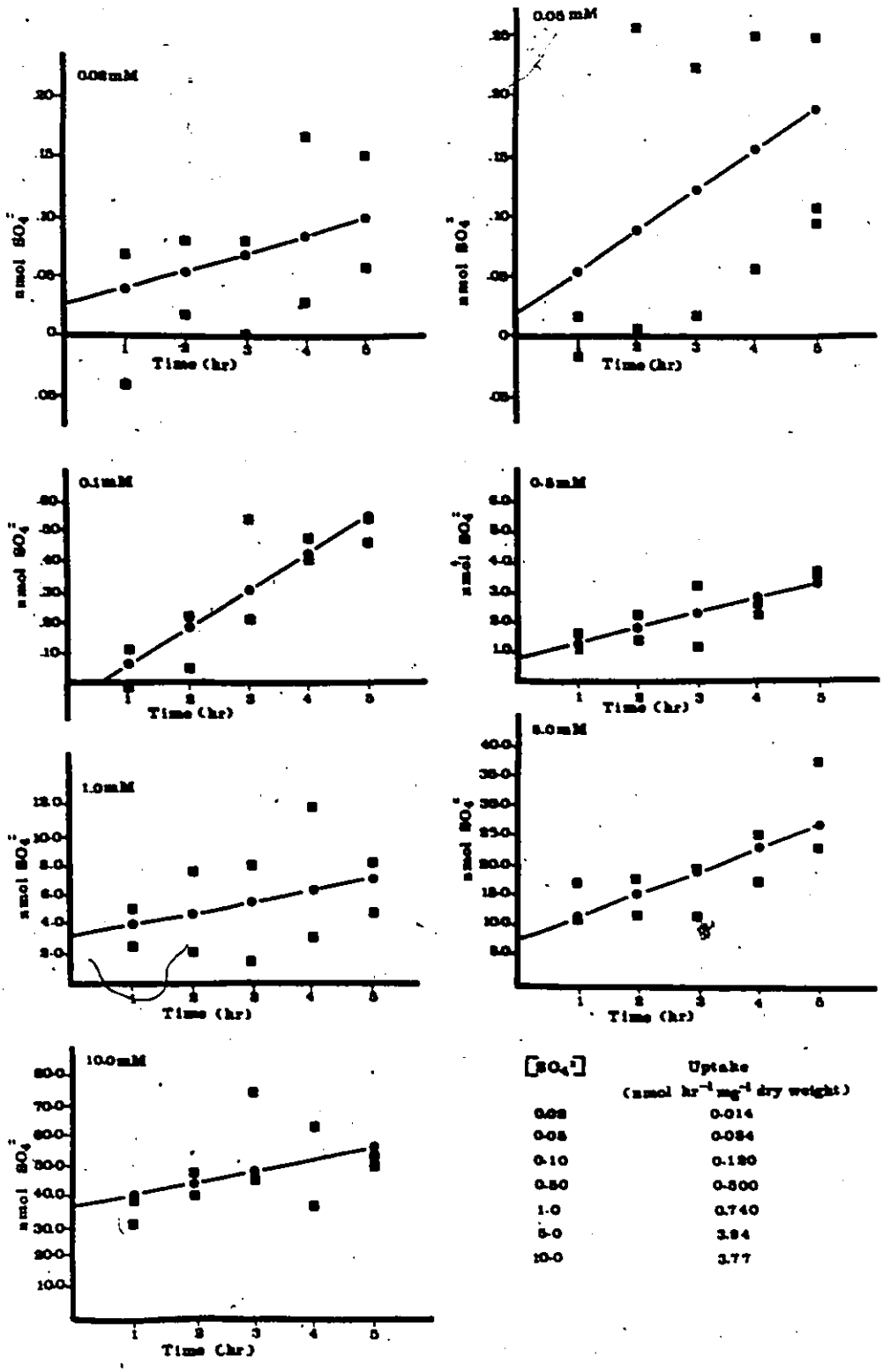

0.10mM
nmol SO4=
Time (hr)
10.0 mM
[SO4=] Uptake
(nmol hr-1 mg-1 dry weight)
0.02 0.02
0.10 0.16
1.0 0.98
10.0 5.32
0.02mM
1.0 mM

Fig. 9 Uptake rate curves for $^{35}SO_4^{2-}$ at 0.02, 0.05, 0.10, 0.50, 1.00, 5.00 and 10.0 mM sulphate (Trial 2). Squares represent duplicate data points and circles represent best fit points.



$[SO_4^{=}]$	Uptake (nmol hr^{-1} mg^{-1} dry weight)
0.05	0.014
0.05	0.084
0.10	0.180
0.50	0.800
1.0	0.740
5.0	3.84
10.0	3.77

a K_m apparent could be determined (Fig. 10). In Trial 2 a K_m apparent could be determined using the range 0.1, 5.0 mM (Fig. 9). Trial 1 gave a K_m apparent of 3.1 mM while Trial 2 gave a value of 2.7 mM. V_{max} values were 3.9 and 2.99 nmol $hr^{-1}mg^{-1}$ dry weight in Trials 1 and 2 respectively (Fig. 10).

The effect of sulphate level on uptake of sulphate by T_4 gametophyte after 48 hr. of labelling is shown in Table 17. It is immediately obvious that concentration had a profound effect on sulphate uptake not only initially but also over a prolonged labelling period. The "counts available" figures reflect the difference in total radioactivity added, necessitated by the wide range of concentrations tested. The cpm x 10^{-4}/mg dry weight (a) is not corrected for differences in specific activity of ^{35}S added but these values are included to show the similarity in actual counts involved. Values given in (b) represent the corrected values. Table 17B shows the ratios of uptake between different concentrations. These comparisons will be discussed later.

There is also a relationship between sulphate concentration and sulphate incorporation into carrageenan fractions (Table 18). The relationship however is not a direct one whereby a given increase in sulphate concentration produces an equivalent increase in incorporation. Although counts incorporated decreased with increasing sulphate concentration, there were not the 10-fold decreases which might be anticipated for a direct relationship. "Counts available" figures are the same as in Table 17.

Other compounds were tested to determine whether incorporation of

Fig. 10 Double reciprocal plots of sulphate concentrations versus velocity of uptake over the sulphate concentration range of 0.10 to 10.0 mM.

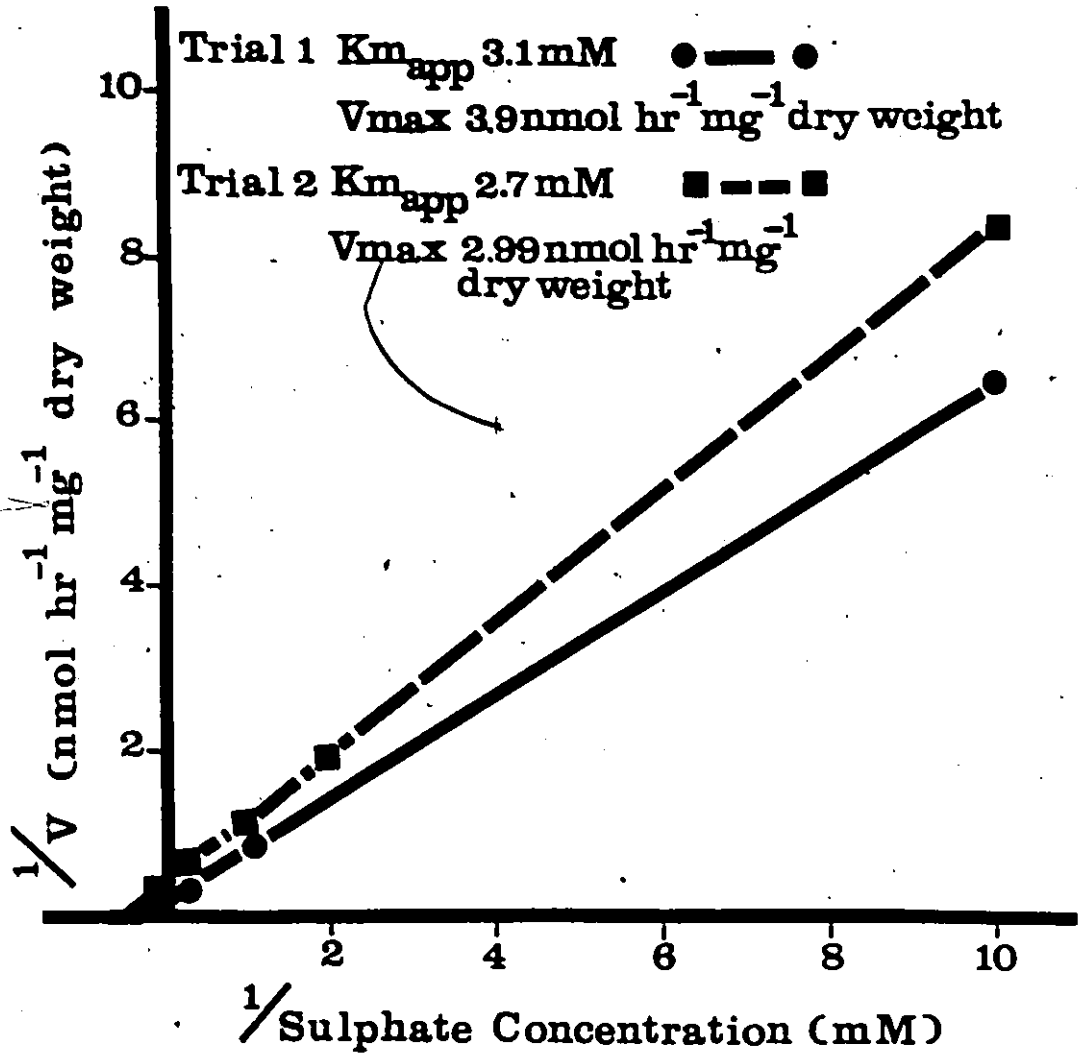

Trial 1 Km_{app} 3.1mM
Vmax 3.9nmol hr^{-1} mg^{-1} dry weight
Trial 2 Km_{app} 2.7mM
Vmax 2.99nmol hr^{-1} mg^{-1} dry weight
1/V (nmol hr^{-1} mg^{-1} dry weight)
10
8
6
4
2
2
4
6
8
10
1/Sulphate Concentration (mM)

Table 17. Sulphate Uptake after 48 hr at Different Sulphate Concentrations*

A. Total uptake $^{35}SO_4^{2-}$

	Sulphate Concentration (mM)			
	0.02	0.10	1.00	10.0
Counts available	3.75	3.81	5.88	8.10
($cpmx10^{-7}$)	±0.099	±0.009	±0.060	±0.045
Uptake				
a) $cpmx10^{-4}$/	1.09	1.13	1.65	1.49
mg dry weight	±0.13	±0.09	±0.02	±0.05
b)nmol SO_4^{2-}/	0.177	0.911	8.55	55.9
mg dry weight	±0.017	±0.074	±0.19	±2.20

B. Comparison of Sulphate Uptake at Various Sulphate Levels

Parameter of Comparison	Concentrations Compared		
	0.1/0.02	1.00/0.10	10.0/1.00
Counts available	0.996	1.57	1.43
	±0.035	±0.03	±0.03
$cpmx10^{-4}$/			
mg dry weight	1.07	1.47	0.904
	±0.21	±0.14	±0.041
nmol/mg dry	5.25	9.45	6.55
weight	±0.91	±0.96	±0.41

* Duplicate samples

Conditions: Light 320 ft-c, 16: 8h:: light:dark; 17 C.

Table 18. ^{35}S Incorporation in Various Fractions from T_4 Gametophyte Labelled in Different Sulphate Concentrations *

A. Total Counts (cpm x 10^{-2}/mg dry weight)

	Sulphate Level (mM)			
Fraction	0.01	0.10	1.00	10.0
Acetone-ethanol soluble	0.698 ±0.15	0.523 ±0.07	0.358 ±0.05	0.068 ±0.01
KCl soluble carrageenan	20.2 ±2.0	14.9 ±1.0	7.33 ±1/9	1.37 ±0.01
KCl insoluble carrageenan	7.26 ±0.67	4.94 ±1.1	3.06 ±0.27	0.24 ±0.03

B. Specific Activities of Carrageenan Fractions (cpm x 10^{-4}/mg carrageenan)

	Sulphate Level (mM)			
Fraction	0.01	0.10	1.00	10.0
KCl soluble carrageenan	2.43 ±0.36	2.24 ±0.14	1.20 ±0.15	0.19 ±0.00
KCl insoluble carrageenan	0.32 ±0.03	0.24 ±0.04	0.15 ±0.02	0.01 ±0.00

* Duplicate samples.

Conditions: Light 320 ft-c, 16: 8h:: light:dark; 17 C.

sulphate into carrageenans would be altered. Nitrate was tested because of the reported effects of fixed nitrogen sources (53) and molybdate because of the reported competitive inhibition of incorporation into Porphyridium sulphated polysaccharide (63). The effect of nitrate and molybdate on incorporation of sulphate into T_4 carrageenans is shown in Table 19. Rather than producing the anticipated inhibitions, these compounds seemed to have no effect on incorporation of sulphate into carrageenan fractions. Both nitrate and molybdate seemed to cause increased levels of ^{35}S label in the acetone-ethanol fraction.

Other fixed nitrogen sources (NH_4Cl and urea) and another Group VI anion (WO_4^{2-}) were tested also for effect on sulphate incorporation into carrageenan (Table 20). As had been observed with MoO_4^{2-}, WO_4^{2-} had virtually no effect on the distribution of ^{35}S incorporated into T_4 gametophyte. The fixed nitrogen sources tested showed some effect. Although NH_4Cl had little effect on incorporation into KCl soluble carrageenan it depressed labelling of KCl insoluble carrageenan. Urea on the other hand caused very much less labelling of KCl soluble carrageenan.

Specific activity values also reflect these differential effects of fixed nitrogen sources. Labelling of both carrageenan fractions appeared depressed in the presence of NH_4Cl with the effect on the KCl insoluble fraction being more dramatic. In the presence of urea the specific activity of the KCl insoluble fraction was unchanged or slightly enhanced while the KCl soluble specific activity value was dramatically lower than control values. WO_4^{2-} appeared to depress slightly the value of the KCl soluble fraction.

Table 19. Effect of Nitrate and Molybdate on ^{35}S Incorporation into T_4 Gametophyte (cpm x 10^{-2}/mg dry weight)*

	Control	5×10^{-2}M NO_3^-	10^{-3}M MoO_4^{2-}
Acetone-ethanol soluble	60.3±5.6	92.2±8.6	90.1±0.9
KCl soluble x Cetavlon precipitate	18.5±6.5	20.5±6.8	16.4±1.0
KCl insoluble x Cetavlon precipitate	35.7±2.4	37.9±14.1	51.9±12.4

* Duplicate samples.

x Extracts were fractionated in 3M KCl and Cetavlon precipitates were dried without decomplexing.

Conditions: Light 320 ft-c, 16:8h:: light:dark; 17 C; $Na_2{}^{35}SO_4$ (0.1 mM) 1 µCi/ml medium; 48h labelling.

Table 20. The Effect of WO_4^{2-} and Fixed Nitrogen Sources on Sulphate Incorporation in T_4 Gametophyte after 48 hr Labelling

A. Total Incorporation (cpm x 10^{-4}/mg dry weight) ++

	Control	10 mM WO_4^{2-}	1mM NH_4Cl *	1mM urea *
Acetone-ethanol soluble	0.119 ±0.02	0.128 ±0.01	0.127	0.150
KCl soluble carrageenan	0.07 ±0.01	0.05 ±0.01	0.04	0.004
KCl insoluble carrageenan	0.08 ±0.01	0.08 ±0.01	0.01	0.10

B. Specific Activities of Carrageenan Fractions (cpm x 10^{-4}/mg dry weight)

	Control	10mM WO_4^{2-}	1mM NH_4Cl	1mM urea
KCl soluble carrageenan	1.34 ±0.13	0.95 ±0.15	0.39	0.08
KCl insoluble carrageenan	0.30 ±0.03	0.32 ±0.04	0.06	0.40

++ Values represent the mean ± the variation from the mean. Where no variation is indicated duplicates were not used.

* Fixed nitrogen concentrations refer to concentration on a nitrogen basis.

Conditions: Light 320 ft-c, 16:8h:: light:dark; 17 C; $Na_2{}^{35}SO_4$ (0.1mM) 1 µCi/ml medium.

Incorporation of sulphate into crude KCl soluble and insoluble fractions Table (21) also differed in the three strains. T_4 was the most efficient in ^{35}S incorporation, although little difference was seen in the KCl insoluble fraction between T_4 and White 16. Blue 7 and White 16 showed equivalent labelling of KCl soluble fractions (both lower that T_4). The level of incorporation of ^{35}S into the KCl insoluble fraction was also lower in Blue 7.

Incorporation of ^{14}C into crude KCl soluble and insoluble carrageenan fractions was not significantly different among the three strains. (Table 21)

Several compounds and a "dark" treatment (samples wrapped in foil) were also tested for effects on net ^{14}C uptake and incorporation. None of the compounds tested had any effect on net uptake of ^{14}C (Table 22). Only the "dark" treatment had a strong effect on net ^{14}C uptake.

Similarly "dark" conditions had a profound effect on ^{14}C incorporation into C. crispus fractions (Table 23). Single samples were tested in this screening experiment. Other conditions tested enhanced incorporation into KCl soluble carrageenans while inhibiting incorporation into KCl insoluble carrageenans. When specific activities are considered (Table 23B), 0.5% galactose appeared to inhibit incorporation into both soluble and insoluble fractions, reflecting a somewhat higher content of KCl soluble carrageenan in this sample.

Some inhibition of ^{14}C incorporation into 3M KCl soluble and

Table 21. Incorporation of ^{35}S and ^{14}C into Three strains of C. crispus after 5 hr of Labelling (cpmx10^{-2}/mg dry weight)

	^{35}S		^{14}C	
	KCl soluble	insoluble	soluble	insoluble
T_4	0.183±0.03 *	1.38±0.34	5.14±0.86	23.0±6.1
Blue 7	0.116±0.01	0.15±0.52	5.61±1.1	22.1±3.1
White 16	0.120±0.02	0.76±0.37	3.27±1.0	18.1±2/9

* All values represent the mean of 2 samples ± variation from the mean.

Conditions: Light 320 ft-c, 16:8h:: light:dark; 17 C; $Na_2{}^{35}SO_4$ (0.1mM) 1 μCi/ml medium.

Table 22. Effect of Various Compounds on Net ^{14}C Uptake by *C. crispus* (per cent of control*)

	6 hr	24 hr	48 hr
"dark" x		40.3	64.0
10^{-6}M 2,4-D x		97.9	101.4
2 mM Na NO_3 x		89.2	88.4
0.5% galactose x		102.1	98.8
5×10^{-2}M Na NO_3	87.4	89.2	84.8
10^{-3}M Na_2 MoO_4	90.2	94.8	88.7

* Based upon mean values. Because of variation differences less than 16% were not considered to be significant.

x Single samples

Conditions: Light 320 ft-c, 16:8h:: light:dark; 17 C; $NaH^{14}CO_3$ (2.36 mM) 1 μCi/ml medium, T_4 gametophyte.

Table 23. Effect of Various Labelling Conditions on the Incorporation of ^{14}C into C. crispus T_4 Fractions (cpm x 10^{-4}/mg dry weight) after 96 hr *

Fraction	Labelling Conditions				
	Control	"Dark"	2mM $NaNO_3$	10^{-6}M 2,4-D	0.5% galactose
Acetone-Ethanol-Soluble	4.69±1.03	0.0904	3.54	3.16	3.56
KCl soluble carrageenan	0.110	0.00849	0.281	0.372	0.287
KCl insoluble carrageenan	0.439	0.00276	0.287	0.264	0.226

B. Specific Activities of T_4 Carrageenan Fractions Labelled under Various Conditions (cpm x 10^{-4}/mg carrageenan)

	Control	"Dark"	2mM $NaNO_3$	10^{-6}M 2,4-D	0.5% galactose
KCl soluble carrageenan	6.60	0.177	7.35	7.05	4.90
KCl insoluble carrageenan	1.73	0.01	1.34	1.10	1.01

* With exception of control (duplicate), single samples were used. Conditions: Light 320 ft-c, 16:8h:: light:dark; 17 C; $NaH^{14}CO_3$ (2.36 mM) 1 μCi/ml medium, T_4 gametophyte.

insoluble carrageenan fractions (Table 24) was shown with nitrate and molybdate. In the presence of $5x10^{-2}$M NO_3^-, T_4 incorporated less ^{14}C into both KCl soluble and insoluble fractions. Although MoO_4^{2-} inhibited incorporation into the KCl soluble fraction, incorporation of ^{14}C into the KCl insoluble fraction was similar to control values. Neither compound appeared to alter the level of ^{14}C in the acetone-ethanol fraction.

3. Time Sequence of Incorporation and Pulse-chase Experiments

Preliminary "pulse-chase" experiments (Table 11 a & b) indicated that ^{35}S label in the KCl soluble carrageenan fraction was in a dynamic state. Both in total counts and specific activities other fractions showed little or no change in ^{35}S or ^{14}C labelling. In order to design an experiment to follow ^{35}S and ^{14}C over a short labelling period followed by a relatively long chase period, knowledge of the timing required to obtain significant labelling of carrageenans was required. A good level of labelling with both ^{14}C and ^{35}S was obtained by 6 hr (Tables 25 & 26). To determine the minimum time necessary for a pulse-chase experiment a short experiment was set up using the Method IV extraction procedure. Results shown in Table 27 are for the "crude" fractions (before CPC precipitation) and therefore the soluble fraction presumably contains free sulphate and ^{14}C counts would include those incorporated into floridean starch.

Both ^{14}C and ^{35}S labelling of the KCl fraction showed a consistent increase in amount from 2 to 3.5 hr. In the KCl insoluble fraction both ^{14}C and ^{35}S label was apparent with 2 hr. However, while ^{14}C labelling was quite

Table 24. Effect of Nitrate and Molybdate on ^{14}C Incorporation in T_4 C. crispus (cpm x 10^{-2}/mg dry weight) *

	Control	5 x 10^{-2}M NO_3^-	10^{-3}M MoO_4^{2-}
Acetone-ethanol soluble	1270±100	1100±40	1150±120
KCl soluble x Cetavlon Precipitate	22.3±3.3	15.5±0.6	13.6±2.6
KCl insoluble x Cetavlon precipitate	27.5±5.1	17.3±1.4	24.6±8.7

* Duplicate samples

x Extracts were fractionated in 3M KCl and Cetavlon precipitates were dried without decomplexing

Conditions: Light 320 ft-c, 16:8h:: light:dark; 17C; $NaH^{14}CO_3$ (2.36 mM) 1 µ Ci/ml medium, T_4 gametophyte, 48h labelling.

Table 25. Time Course of ^{35}S Incorporation into T_4 Gametophyte (cpm x 10^{-2}/mg dry weight) x

	6 hr	24 hr	48 hr
Acetone-ethanol soluble	7.47±0.21	28.1±4.1	60.3±5.6
KCl soluble * Cetavlon precipitate	1.90±0.14	5.79±3.7	18.5±6.5
KCl insoluble * Cetavlon precipitate	4.22±0.37	14.4±5.5	35.7±2.4

x Duplicate samples

* Extracts were fractionated in 3M KCl and Cetavlon precipitates were dried without decomplexing.

Conditions: Light 320 ft-c, 16:8h:: light:dark; 17 C; $NaH^{14}CO_3$ (2.36 mM) 1 μCi/ml medium, T_4 gametophyte.

Table 26. Time Course of ^{14}C Incorporation in T_4 Gametophyte (cpm x 10^{-2}/mg dry weight) x

	6 hr	24 hr	48 hr
Acetone-Ethanol Soluble	915±25	1140±110	1270±100
KCl soluble * Cetavlon precipitate	10.7±2.1	12.8±0.3	22.4±3.3
KCl insoluble * Cetavlon precipitate	12.8±5.2	13.1±6.2	27.5±5.1

x Duplicate samples

*Extracts were fractionated in 3M KCl and Cetavlon precipitates were dried without decomplexing.

Conditions: Light 320 ft-c, 16:8h:: light:dark; 17 C; $NaH^{14}CO_3$ (2.36 mM) 1 μCi/ml medium, T_4 gametophyte.

Table 27. Time Sequence of Incorporation of ^{35}S and ^{14}C into Carrageenan Fractions in T_4 Gametophyte

A. Incorporation of ^{35}S (cpm x 10^{-2}/mg dry weight)

	2 hr	3.5 hr	5 hr
KCl soluble fraction	7.3 ±0.80	12.3 ±5.5	18.3 ±3.0
KCl insoluble fraction	0.286 ±0.274	0.564 ±0.519	1.38 ±0.34

B. Incorporation of ^{14}C (cpm x 10^{-2}/mg dry weight)

	2 hr	3.5 hr	5 hr
KCl soluble fraction	303 ±8.50	437 ±41.0	514 ±86
KCl insoluble fraction	12.9 ±0.42	21.4 ±1.5	23.0 ±6.1

Conditions: Light 250 ft-c, 16:8h:: light:dark; 17 C; $Na_2{}^{35}SO_4$ (0.1 mM) 1 μCi/ml medium.

consistent between duplicates at all time points, the ^{35}S labelling was not well duplicated until 5 hr. The ^{35}S data confirmed that in future pulse-chase experiments a relatively long chase period might be necessary since uptake rates were similar for all samples (cpm x 10^{-4}/mg dry weight $\bar{X}$= 0.89$^{+}_{-}$ 0.16. $\bar{X}$ is the mean of 6 values $^{+}_{-}$ standard deviation). The data also indicated that $^{35}SO_4^{2-}$ of considerably higher specific activity must be used to yield meaningful results.

Since it was indicated that the precursor of kappa carrageenan was in the 0.3M KCl soluble fraction (Table 11) and it was known that this fraction was a mixture of polysaccharides (47), it was desirable to subfractionate it to give better resolution of the actual precursor fraction. Previous data had shown that the use of 3M KCl in fractionation produced shifts in the labelling pattern of T_4 gametophyte from that expected for the labelling time (Tables 19 and 24). This suggested that a fraction soluble in 0.3M KCl but insoluble in 3M KCl might be significantly labelled. Thus the 0.3M KCl soluble fraction was subfractionated. To characterize the two subfractions, a large extraction of T_4 gametophyte material was done and fractions were characterized by infrared analysis and immunodiffusion.

Fig. 11 shows infrared spectra of (from top to bottom) 0.3M KCl soluble - 3M KCl insoluble (before CPC precipitation), 0.3M KCl soluble (unfractionated), and 0.3M KCl insoluble (kappa) carrageenans. Some differences are apparent immediately - especially the peak at 805 cm^{-1}. Fig. 12 shows (from top to bottom) infrared spectra of 0.3M KCl soluble (unfractionated), 0.3M KCl soluble-3M KCl insoluble, and 0.3M KCl soluble-3M KCl soluble carrageenan

Fig. 11 Infrared spectra of (from top to bottom) 0.3M KCl soluble-3M KCl insoluble, 0.3M KCl soluble (unfractionated), and 0.3M KCl insoluble (kappa carrageenans from T_4 gametophyte.

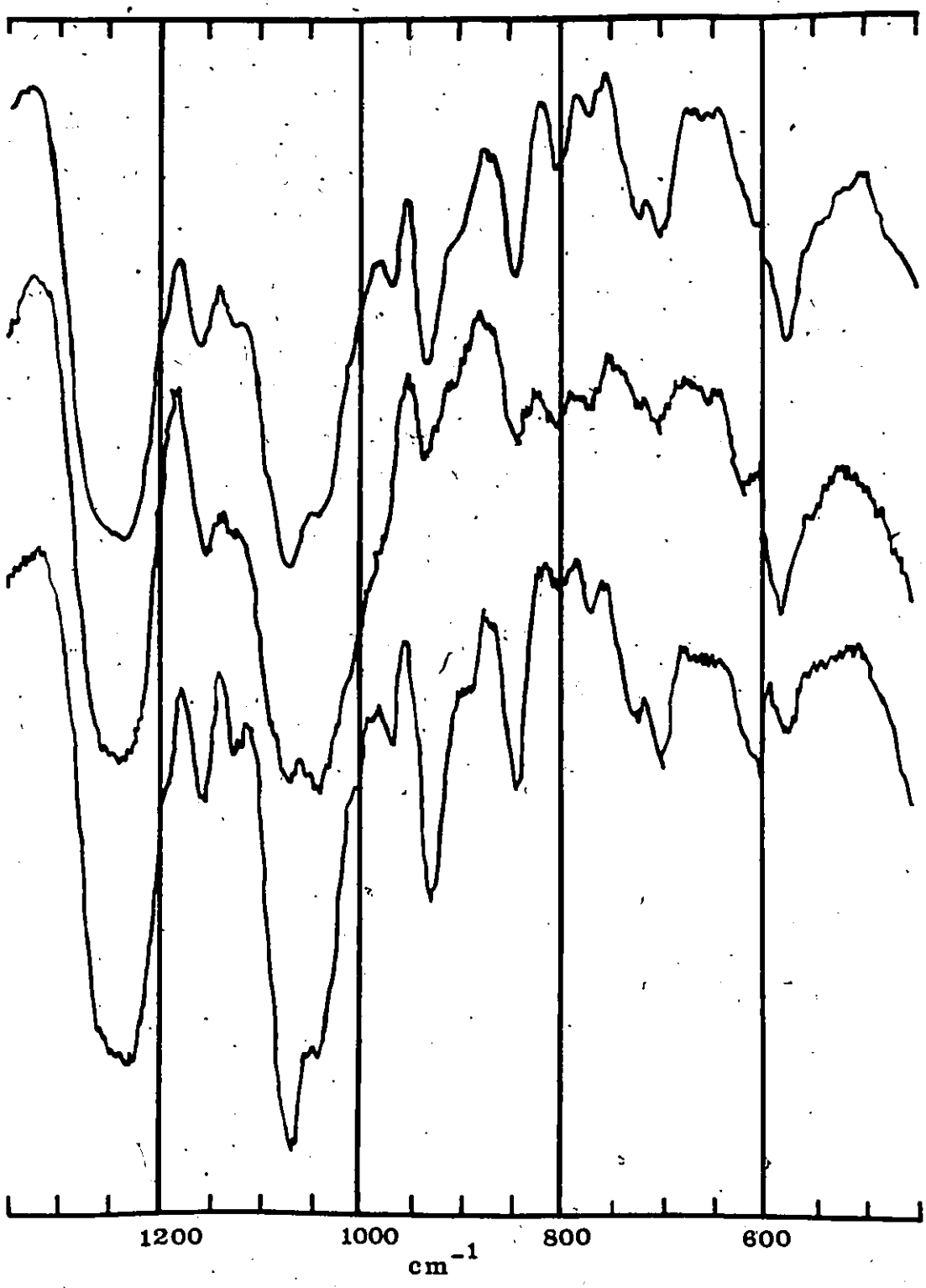
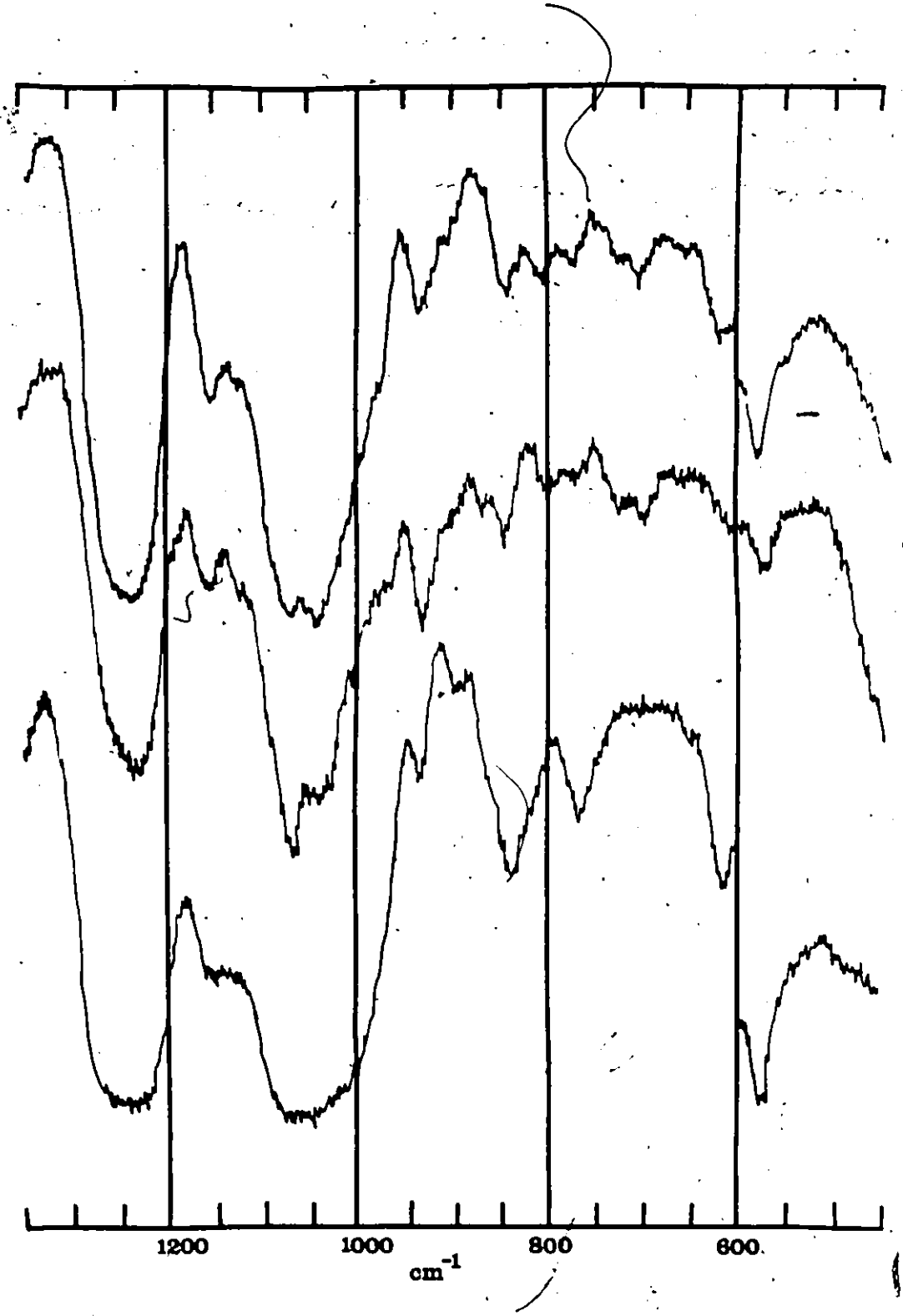

1200
1000
800
600
cm^{-1}

Fig. 12 Infrared spectra of (from top to bottom) 0.3M KCl soluble (unfractionated), 0.3M KCl soluble3M KCl insoluble, and 0.3M KCl soluble-3M KCl soluble carrageenans from T_4 gametophyte.

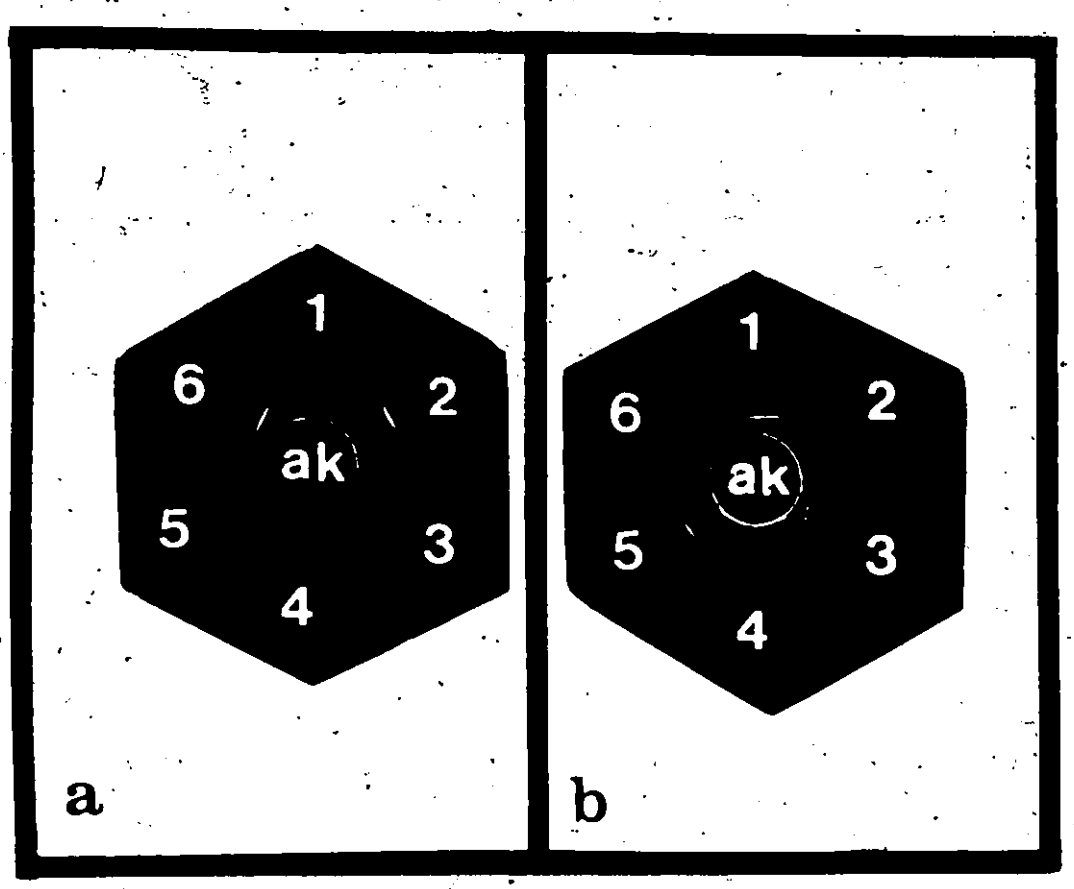

1200
1000
800
600
cm-1

fractions. As well as the peak at 805 cm^{-1} it is obvious that differences exist at the area of characteristic sulphate peaks (810 - 850 cm^{-1}). It would seem that the 3M KCl soluble fraction has a distinctly different pattern of sulphation.

Fig. 13 shows immunodiffusion plates with the various fractions and standard carrageenan samples run against anti-kappa carrageenan antiserum. Differences in precipitability with different fractions are obvious. T_4 3M KCl soluble and Eucheuma spinosum 0.3M KCl soluble show much lower reactivity. T_4 0.3M KCl insoluble (kappa) carrageenan shows a reaction of identity with the kappa standard confirming infrared and chemical evidence. T_4 kappa also shows an identity reaction (or at least partial identity) with the outer band and of partial identity with the inner band of the iota sample ①. The junction lines with iota (0.3M KCl soluble carrageenan from E.spinosum) and 3M KCl insoluble are very faint but it appears that at least partial identity exists between 3M KCl insoluble and the outer iota band. Lines with T_4 3M KCl soluble and E. spinosum 0.3M KCl soluble were very faint. In plate b a reaction of partial identity is seen with kappa standard ① and 3M KCl insoluble ②. Similarly a reaction of partial identity is seen between iota outer band (3) and the 3M KCl insoluble ② and 3M KCl soluble ④. The 3M KCl soluble also shows a reaction of partial identity with 0.3M KCl soluble (unfractionated) ⑤ as anticipated. The 0.3M KCl soluble carrageenan from E. spinosum showed faint precipitin lines with the antiserum but the two lines seen with iota exist.

Pulse-chase experiments were then undertaken to gain some information about the kappa carrageenan "precursor". Chemical considerations restrict the possibilities for precursors. Early precursors of carrageenans such as

Fig. 13 Immunodiffusion plates of various carrageenan fractions with anti-kappa carrageenan antiserum. Carrageenan fractions in a are: (1) iota from E. spinosum; (2) 0.3M KCl soluble-3M KCl insoluble from T_4; (3) 0.3M KCl soluble-3M KCl soluble from T_4; (4) 0.3M KCl soluble from E. spinosum; (5) kappa standard; (6) 0.3M KCl insoluble from T_4. In plate b the fractions are: (1) kappa standard; (2) 0.3M KCl soluble-3M KCl insoluble from T_4; (3) iota from E. spinosum; (4) 0.3M KCl soluble-3M KCl soluble from T_4; (5) 0.3M KCl soluble (unfractionated) from T_4; (6) 0.3M KCl soluble from E. spinosum.


1
6
2
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b

activated sulphate (adenosine 5' - phosphosulphate (APS)) and galactose would be present in the acetone-ethanol fraction. More immediate precursors sharing some chemical similarities would be found in the 0.3M KCl soluble fraction as no other fraction with suitable structure to serve as an immediate precursor was found in C. crispus (4).

Table 28 shows the specific activity values of the various fractions after a 1.5 hr pulse (^{35}S 5.2 x 10^7 cpm/μmol sulphate; ^{14}C 3.0 x 10^6 cpm/μmol bicarbonate) and prolonged chase. Unfortunately the yields of KCl insoluble carrageenans were very low in some samples and thus it was not possible to use duplicate values. It is evident however that specific activity values were much higher in the 0.3M KCl soluble fractions than those of the insoluble fractions. More significantly the shifts in values were different in the soluble and the insoluble fractions.

Because of the problems encountered with yields in the first experiment, another T_4 sample was used to repeat the pulse-chase experiment (^{35}S 7.5 x 10^7 cpm/μmol sulphate; ^{14}C 3.6 x 10^6 cpm/μmol bicarbonate). The specific activity values are given in Table 29. It is again obvious that specific activity values of the 0.3M KCl soluble fractions greatly exceeded those of the insoluble. One half of the 0.3M KCl soluble fraction was subfractionated and thus significant amounts of 0.3M KCl soluble-3M KCl soluble and 0.3M KCl soluble 3M KCl insoluble carrageenans could be extracted. In this experiment the level of incorporation was considerably lower than in the earlier one, possibly because of the problem of temperature regulation initially or other growth status

factors (plants were cultured originally at a much lower density prior to shipping (Brian Ives, personal communication)). Nevertheless, specific activity values were much higher for the 0.3M KCl soluble fractions.

Table 28. Specific Activity of Carageenan Fractions in T_4 Gametophyte During a 1.5 hr Pulse and 48 hr Chase (cpm x 10^{-4}/mg galactose equivalent) [x]

A. Pulse with ^{35}S

	Time				
	0	1.5 hr*	2 hr_c*	24 hr_c	48 hr_c
0.3M sol[+] (unfractionated)	0.011	0.406	0.429	0.357	0.084
0.3M sol-3M insol[+]	5.50	25.3	0.95	12.1	25.9
0.3M insol	0.015	0.052	0.062	0.135	0.218
Acetone-ethanol[+] sol	0	0.016	0.024	0.012	0.004

B. Pulse with ^{14}C

	Time				
	0	1.5 hr_p	2 hr_c	24 hr_c	48 hr_c
0.3M sol (unfractionated)	0.126	2.80	2.91	3.22	4.39
0.3M sol-3M insol	0.16	22.7	23.5	17.0	16.4
0.3M insol	0.014	0.248	0.113	0.303	0.915
Acetone-ethanol sol	0.056	4.49	4.62	1.75	0.72

X Single samples

* Subscript p refers to pulse time and subscript c refers to chase time

+ The abbreviations sol refers to KCl soluble fraction and insol refers to KCl insoluble fraction.

The acetone-ethanol soluble counts refer to cpm x 10^{-4}/mg dry weight of plants.

Conditions: Light 220 ft-c, 16:8h:: light:dark; 17 C; $Na_2{}^{35}SO_4$ 5.2 x 10^7 cpm/μmol sulphate; $NaH^{14}CO_3$ 3.0 x 10^6 cpm/μmol bicarbonate.

Table 29. Specific Activity of Carrageenan Fractions in T_4 Gametophyte During a 1.5 hr Pulse and 96 hr Chase (cpm x 10^{-2}/mg galactose equivalent) [x]

A. Pulse with ^{35}S

	Time				
	0	1.5 hr_p	2 hr_c [*]	48 hr_c	96 hr_c
0.3M sol[+]	-0.25	2.13	3.14	3.27	6.10
(unfractionated)	±0.01	±0.67	±0.52		
0.3M sol-	0.44	3.21	3.38	4.43	6.54
3M sol	±0.30	±0.03	±0.37	±1.4	±2.1
0.3M sol-	0.084	1.15	1.36	0.90	1.90
3M insol [+]	±0.03	±0.77	±0.95	±0.56	±0.09
0.3M insol	0.041	0.18	0.29	0.40	1.26
	±0.04	0.04	±0.14	±0.24	±0.28
Acetone-[#]					
Ethanol					
sol	0.06	0.12	0.22	0.23	0.60
	±0.0	±0.03	±0.01	±0.01	±0.04

B. Pulse with ^{14}C

	Time			
	0	1.5 hr_p	2 hr_c	96 hr_c
0.3M sol				
*unfractionated)	0.49	80.1	52.7	168
	±0.01	±9.3	±18	±31

0.3M sol-	0.53	58.2	45.6	237
3M sol	±0.11	±7.7	±3.4	±3.6
0.3M sol-	0.15	27.9	10.7	88.3
3M insol	±0.16	±2.0	±5.0	±13
0.3M insol	0.09	6.72	3.72	41.7
	±0.01	±1.5	±0.80	±9.5
Acetone-				
ethanol-	0.70	596	450	517
sol	±0.14	±51	±23	±20

x Duplicate samples

*Subscript p refers to pulse time and subscript c refers to chase time

+ The abbreviation sol refers to KCl soluble fraction and insol refers to KCl insoluble fraction at that concentration.

The acetone-ethanol soluble counts refer to cpm x 10^{-2}/mg dry weight of plants.

Conditions: Light 250 ft-c, 16:8h:: light:dark; 17C; $Na_2{}^{35}SO_4$ 7.5 x 10^7 cpm/µmol sulphate; $NaH^{14}CO_3$ 3.6 x 10^6 cpm/µmol bicarbonate.

DISCUSSION

I. ANALYTICAL METHODS

1. Sulphate determination

Many previous methods for the determination of inorganic sulphate are tedious and time-consuming, are sensitive to small quantities of many common reagents, or involve the use of unstable and/or carcinogenic reagents. Most of these methods depend upon barium sulphate formation with subsequent determination of this compound or the original barium partner. Of all previously described methods, turbidometric methods such as that of Dodgson (20, 21) remain the simplest and most rapid. However, Dodgson's gelatin reagent requires great care in preparation and is relatively unstable (20, 21). Several reagents have been employed as mechanical support in precipitation analysis (8, 18, 43). The assay developed here using agarose as a stabilizing agent overcomes the problems associated with meticulous reagent preparation and reagent instability.

The values obtained using the turbidometric assay agree closely with those obtained using the Jones and Letham (33) method. The turbidometric method however is simpler, less time-consuming, and eliminates the manipulation

of a carcinogenic substance. The lack of effect of $MgCl_2$ even at a reasonably high concentration suggests that the turbidometric method is insensitive to divalent cations unlike the barium chloranilate method (36). Also the ability to determine sulphate in the presence of protein by the use of the turbidometric protein and sulphate assays in tandem makes the assay particularly useful for determination of inorganic sulphate in protein solutions. The sensitivity of the sulphate assay permits detection of very small amounts of inorganic sulphate in the presence of relatively large quantities of protein. This aspect would be of particular importance in enzyme preparations in which the enzyme is sensitive to small amounts of ammonium sulphate.

2, Protein determination

The turbidometric protein assay using the agarose reagent used for sulphate analysis without the addition of $BaCl_2$ overcomes many problems associated with previously described methods. The protein assay is simple and the stability of turbidity over a long period of time recommends the method for large sample numbers. Linearity of the assay over an hundred-fold concentration range (25 - 2500 µg BSA equivalents) makes the assay useful for determining protein in unknown samples having very variable protein levels. Also the large sample volume allows quantitation of relatively dilute samples without concentrating procedures. This aspect as well as the wide sensitivity range suggest the assay to be suitable for monitoring column effluents, etc. where the presence of other ultraviolet absorbing substances may preclude monitoring at O.D. 280 nm.

Lack of interference by amino acids and mercaptoethanol and the stability of the turbidity give this method advantages over that of Lowry et al. (40) As with other protein assays, different proteins give different values per mg total protein. The apparent linearity of optical density versus concentration with other proteins suggests that a conversion factor may be used to compare values determined by other methods. Thus any protein precipitable by TCA should be determinable by the simple and rapid turbidometric protein assay.

II. CULTURING

Conditions used for C. crispus culturing were adapted from those previously reported for in vitro assessment of various parameters in excised vegetative (9), germling and/or mature tissue (11, 44), or mass culturing of vegetative and reproductive plants (53). Conditions were chosen to give good carrageenan biosynthesis while preventing rapid nutrient depletion because of overgrowth. The results of such overgrowth were seen with the rapidly growing T_4 gametophyte (Table 7). High light and good nutrient conditions resulted in bleaching and fragmentation of the plants within 24 hr. In the normal environment of C. crispus loss of red pigmentation is normal during the summer months, especially in warmer waters (53, 71). Similar bleaching can be induced in mass cultures by re-circulation of seawater and increased temperatures (53). Fertilization of such plants resulted in algae with higher nitrogen and moisture contents but lower dry matter and carrageenan.

The term "Neish" effect has been used to describe the effect of

nitrogen deficiency under otherwise optimal growth conditions. In this situation there is bleaching and enhanced accumulation of dry matter and sulphated polysaccharides in C. crispus. In the case of the described T_4 cultures (Table 7) bleaching could not be prevented or reversed by the addition of fixed nitrogen sources alone. It appeared that depletion of other nutrients during overgrowth prevented reversal. Fragmentation was evident in plants previously grown in media unsupplemented with extra fixed nitrogen compounds but not in the controls (no pretreatment). Phosphate has been implicated in maintenance of the intact thallus (53). The presence of phosphate lessened spontaneous fragmentation (53). Since the rapidly growing strain is prone to such spontaneous fragmentation and grows vegetatively via this process (52) it is possible that phosphate plays an important rule in growth control.

III. LABELLING STUDIES

1. Sulphate uptake

Because of differences in the degree of sulphation of the major polysaccharides in gametophytic and sporophytic C. crispus plants and the extremely large amounts of these sulphated polysaccharides present one might anticipate very different quantitative requirements for sulphate which could be reflected in uptake differences. Despite these polysaccharide differences, however, the uptake of sulphate appeared to be unrelated to karyotype (Fig. 7 and Results III 1.1). Previous work with Fucus, a brown alga, indicated that the relationship between sulphate uptake and internal requirement for sulphate was

not a direct one (5). The results reported here (Results III 1.1 and 2) indicate that "growth status" may be a more important factor in determining sulphate uptake than ploidy level. Plant growth status would be influenced by the complex interaction of a number of factors such as temperature, light (intensity and duration), and medium enrichment. All of these factors have been reported to influence growth and/or carrageenan production in C. crispus (7, 9, 26). Other factors implicated in this thesis are plant strain and/or in vitro culture conditions (Tables 8, 16B). The composition of the labelling medium may also be an important influence. Under the conditions used here sulphate uptake values exceeded those reported by Loewus et al for C. crispus (39) and Evans et al (23) for Laminaria hyperborea (a brown alga) over the same time period by several orders of magnitude for similar amounts of plant material. Since non-cultured material and a more complex labelling medium (23, 39) were used in both cases, these aforementioned factors (culture conditions and labelling medium) one or both may be involved in the observed differences as well as differences in the algae used.

The effect of different light intensities on cultured or non-cultured gametophytic material appeared to be complex over a prolonged labelling period (Table 8). Although cultured material and T_4 gametophytic material showed a somewhat enhanced uptake under higher light intensities, the non-cultured material showed increased uptake under lower light conditions, at least from 48 hr on. Differences are not great enough to draw quantitative conclusions but it would appear that qualitatively the cultured material reacted in a manner more similar to the rapidly growing T_4 gametophyte than the non-cultured

gametophyte. In sulphate incorporation into carrageenan the cultured gametophyte also appeared to be intermediate between non-cultured and T_4 samples. This observation is supported by apparent shifts in the quality of carrageenan fractions (Table 10).

Thus the uptake of sulphate in C. crispus is complex and may not be directly related to an immediate internal requirement, as Bidwell reported with Fucus (4). There was a rapid initial uptake at most concentrations of sulphate followed by a period of linear uptake at a reduced rate (Figs. 8 & 9). It would appear that, as in higher plants (55), sulphate uptake is multiphasic with different kinetic constants applying at different concentration ranges, since negative apparent V_{max} values were obtained with double reciprocal plots using all concentrations or the lower levels. As noted by Nissen (55), extrapolation from one phase to another "results in meaningless double reciprocal plots often with negative values for V_{max}". An apparent phase shift in C. crispus takes place between 0.02×10^{-5} and 10^{-4}M (Results III 2.). It is interesting that this is the range of such a shift in higher plants (10^{-5} - 10^{-4}M) (55).

The apparent K_m determined, $2.9 \pm 0.2 \times 10^{-3}$M, is somewhat higher than the range estimated for many higher plants and other organisms of 10^{-5} to 10^{-4}M (39) and substantially higher than that reported for the fresh water alga Porphyridium aerugineum of 2.5×10^{-6}M (66); this might be anticipated on the basis of the lower ambient SO_4^{2-} concentration of fresh water compared to seawater. The K_m apparent is also higher than that found for Fucus serratus by Coughlan (15) of 5.9×10^{-5}M. However Coughlan pre-starved plants for C and S for 24 hr and pre-incubated plants for 30 min at a given sulphate concentration.

These factors and the extremely short time over which he measured uptake (1 hr) could account for differences not accounted for by differences in type of alga tested.

2. ^{14}C Uptake

Despite exchange problems certain characteristics of net ^{14}C uptake by C. crispus could be determined. As in the case of ^{35}S uptake, it would appear that net ^{14}C uptake is related more to the growth status of the plants than to the life cycle stage (Results III. 1.3). Although the similarity in sulphate uptake between sporophyte and gametophyte was perhaps unexpected because of differences in sulphate content of the major polysaccharides, the similarity in net ^{14}C uptake might be anticipated in plants harvested from the same location and cultured under the same conditions and therefore having a similar growth status. Different T_4 uptake values with different samples presumably reflected differences in such growth status, as these samples were all derived from a single clone.

With the exception of the "dark" condition, none of the treatments tested had an appreciable effect on the net uptake of ^{14}C (Table 22). It would appear that none of the compounds tested directly affected the net photosynthetic carbon fixation under the experimental conditions.,

3. Sulphate Incorporation in Sporophyte and Gametophyte

In incorporation studies an estimate of reproducibility of data is of

prime importance when sample numbers must be limited by availability of material and time-consuming extraction procedures. An experiment with $^{35}SO_4^{2-}$ using five replicates per time point was carried out to give such an estimate of reproducibility. Variation on a percent basis ranged from 6 to 40% for the different fractions. Student's t test was employed to test the significant differences. All comparisons were significant ($p < 0.01$) and all but one comparison gave p values of < 0.001. Values compared at different time points were total counts incorporated into 0.3M KCl soluble and insoluble carrageenans, 0.3M KCl soluble - 3M KCl soluble carrageenans, specific activities of 0.3M KCl soluble and insoluble carrageenans, and incorporation into acetone-ethanol soluble fractions. Thus the data are very reproducible and significant differences exist between time points.

Of primary importance in the distribution of ^{35}S and ^{14}C was the life cycle stage of the plants (Tables 9, 12). Sulphate was incorporated into karyotype specific polysaccharides as expected on the basis of chemical analysis (47). In the preliminary experiment comparing haploid and diploid plants (Table 9), the ratios of total ^{35}S counts into soluble/insoluble carrageenans were 36 and 80 in the diploid and 0.7 and 0.2 in the haploid 3W cultures at 48 and 96 hr respectively. Although counts in the soluble fraction in the sporophyte doubled in the 48 to 96 hr interval, counts in the insoluble fraction remained virtually unchanged (Table 9). This fact and later observations suggested that the 0.3M KCl insoluble fraction in the sporophyte was not involved in biosynthesis of the major carrageenan in the sporophyte. Yield differences reported here with different extraction procedures (Results III 1.2) raise the question stated earlier

(47)as to the actual existence of an insoluble fraction in the sporophyte.

In the second comparison experiment (Table 12) the ratios of total counts in 0.3M KCl soluble/insoluble were 3.5 and 2.9 at 24 and 48 hr in the gametophyte while in the sporophyte there was neglibible incorporation into or yield of KCl insoluble carrageenan. In the preliminary experiment all specific activity values appeared to be constant from 48 to 96 hr. In the T_4 gametophyte (Table 15) over the same time period specific activity values increased. The KCl soluble to insoluble ratios in the preliminary experiment suggested the possibility of a shut-down of carrageenan synthesis. The significance of these ratios of specific activities will be discussed more fully in regard to the pathway of biosynthesis (Discussion III 5).

With ^{14}C the ratio of total counts in 0.3M KCl soluble to insoluble were 2.3 ± 0.6 at 24 hr and 2.0 ± 0.4 at 48 hr for the gametophyte. There was negligible yield of or labelling of the KCl insoluble fraction in the sporophyte (Table 15). Thus the gametophyte ratios were double those expected for an equivalent synthesis of 0.3M KCl soluble and insoluble fractions. Specific activity values were stable in both gametophyte and sporophyte (Table 15) over the 24 to 48 hr period. Stability with ^{14}C labelling might be anticipated on the basis of possible randomization of labelling of galactose residues because of the path of ^{14}C fixation and carbohydrate interconversions (73). The Calvin-Benson cycle has been shown to predominate in Iridophycus (3) and apparently in C. crispus (4). Galactose compounds are the major labelled carbohydrates in C. crispus after 4-6 hr of photosynthetic ^{14}C fixation (4). Bidwell (4) found that of

^{14}C taken up 50.1% was in the 50% alcohol soluble fraction. In this fraction 65% of the label was in floridoside (a galactosyl-glycerol compound which is the main storage compound in many red algae (4)). The only other detectable label was in a compound which yielded only galactose upon hydrolysis with HCl. In Iridophycus flaccidum (3) an alga closely related to C. crispus, Bean and Hassid found after 2 hr of photosynthesis, that of the 97% of total label in the 80% alcohol soluble extract 65% was in floridoside.

Sporophytic plants incorporated ^{35}S into lambda carrageenan at a much greater rate than did gametophytes into kappa carrageenan in both comparison experiments (2.0-fold higher at 48 hr and 12-fold higher at 96 hr (Table 9) in the first and 6-fold higher at 24 hr and 5-fold higher at 48 hr in the second comparison experiment (Table 12). Any differences in incorporation were not due to differences in carrageenan content as the actual yields of carrageenans were similar in both types of plants.

The sporophyte also incorporated ^{14}C into lambda carrageenan at a much greater rate than did the gametophyte into kappa carrageenan (approximately 4 times as rapidly based on means (Table 15). If the rates of synthesis of lambda and kappa carrageenans were equivalent one might expect total counts of ^{35}S to be approximately double in lambda because of different sulphation patterns. With ^{14}C however one would expect equivalent incorporation. The ratios obtained are considerably higher than expected. Incorporation into total carrageenans did not show great differences between karyotypes. The conversion of a 0.3M KCl soluble "precursor" to kappa

carrageenan in the gametophyte would account for the time sequence difference. Thus the synthesis of lambda carrageenan would appear to be more direct than that of kappa carrageenan.

4. The Effect of Various Conditions on ^{35}S and ^{14}C Labelling of C. crispus Gametophytes

Growth status seemed to be the determining factor in total amount of sulphate incorporation into carrageenans of gametophytes. Gametophyte plants harvested from different locations and cultured in vitro for different lengths of time showed differences in total labelling and specific activity values of carrageenan fractions (Table 9).

Complex interactions occur between different factors. The effect of light for example appeared to be dependent upon the strain and-or pre-culturing of plant material (Table 10). Culturing shifted "normal" plants toward carrageenan synthesis. The light differences actually had little effect on the ratio of specific activities (soluble/insoluble) in cultured and T_4 gametophytes (11.4 and 13.0 for cultured and 4.96 and 6.33 for T_4 for the high and low light conditions respectively) while the non-cultured material showed an apparent shift in specific activity ratio (23.8 and 14.6 for high and low light respectively).

The level of sulphate in the external medium affected both sulphate uptake and incorporation of sulphate into carrageenan fractions (Tables 17, 18). If the total counts available were equivalent and the rates of incorporation

independent of concentration then the "concentration ratio" (CR) of counts incorporated into carrageenans at various levels of $^{35}SO_4^{2-}$ would be the reciprocal of the concentrations tested, i.e. at 10 mM one would expect the counts incorporated to be 1/10 of the counts incorporated at 1 mM and the CR = 0.1. This ratio provides a convenient reference point for quick determination of concentration effects. Table 30 shows the experimental ratios found with four different $^{35}SO_4^{2-}$ levels. It is immediately obvious that, at lower concentrations, the experimental ratios are much larger than the CR's. The maximum range of counts over a 10^3 concentration range was 15 for the acetone-ethanol soluble fraction, 16 for the 0.3M KCl soluble fraction, and 38 for 0.3M KCl insoluble fractions. Only at the highest concentrations tested did experimental ratios of total counts approach the CR's predicted on the basis of a direct relationship. At the highest levels tested (1.0 and 10.0 mM) the experimental ratio is about equal to the CR in the KCl insoluble carrageenan. This observation suggests that at the higher sulphate levels the saturation level for production of kappa carrageenan has been approached at the time tested. The lower experimental ratio (compared to lower concentrations) with the acetone-ethanol soluble and the KCl soluble carrageenan fractions also suggests that a saturation level is being approached.

Although Ramus (63) found competitive inhibition by MoO_4^{2-} of sulphate uptake and incorporation into sulphated polysaccharides in Porphyridium, Coughlan found no effect with MoO_4^{2-} or WO_4^{2-} in Fucus serratus (15). The results reported here support Coughlan's (15) findings (Tables 21 & 22). Even at 10^{-3}M MoO_4^{2-} and 10^{-2}M WO_4^{2-} no inhibition of $^{35}SO_4^{2-}$ incorporation was

Table 30. Experimental Ratios of ^{35}S Incorporation

(Theoretical ratio 0.1 to next lowest concentration)

	Concentrations compared (mM)		
	0.10/0.01	1.00/0.10	10.0/1.00
Acetone-ethanol soluble	0.809 ±0.28	0.712 ±0.20	0.200 ±0.06
KCl soluble carrageenan	0.750 ±0.12	0.509 ±0.16	0.210 ±0.05
KCl insoluble carrageenan	0.701 ±0.22	0.663 ±0.20	0.079 ±0.02

evident. Although nitrate at relatively high levels also did not alter the incorporation of $^{35}SO_4^{2-}$ into carrageenan fractions, it enhanced labelling in the acetone–ethanol soluble fraction, as did MoO_4^{2-}. In the presence of another fixed nitrogen source, urea, reported to be a poor nitrogen source for C. crispus (53), after 48 hr of labelling there was very little label in the KCl soluble fraction although labelling of the insoluble fraction was similar to control values (Table 11). Conversely in the presence of NH_4Cl the level of label in the KCl insoluble fraction was greatly reduced while label in the KCl soluble fraction was similar to controls. Specific activity values suggest some inhibition of labelling in the KCl soluble fraction also in the presence of NH_4Cl.

The above described differential effect of various fixed nitrogen sources is partially in contrast to the findings of Neish et al (53). It would appear that NH_4Cl obstructed the conversion of a precursor in the KCl soluble fraction into kappa carrageenan. Conversely in the presence of urea conversion of KCl soluble precursor of kappa carrageenan would continue but formation of the precursor would have been substantially impaired during the 48 hr labelling period. It is interesting that Wong and Craigie (80) found no effect on the enzyme activity which converted "μ" carrageenan to kappa carrageenan with NH_4Cl up to 10mM but did find inhibition of activity with 9mM Na_2WO_4 (63%) and 9mM $Na_2Mo\ 0_4$ (32%). Of course sensitivity to substrate analogues in an in vitro assay would be greatly enhanced compared to the in vivo situation. Wong and Craigie found the enzyme activity associated with the particulate fraction, hence accessibility may be a major factor for the substrate analogues. Lack of effect by NH_4Cl suggests the enzyme is probably not the site of action. Rather

it would appear that the effect of various nitrogen sources is a general growth status effect.

The "dark" treatment, as might be expected, had a dramatic effect on the incorporation of ^{14}C. Thus as reported earlier there is net ^{14}C fixation in the dark (16), but net photosynthetic carbon fixation is the predominant source of carbon for carrageenan production. Although other treatments showed relatively little effect on total incorporation into carrageenans, it would appear that all tended to shift labelling in the direction of the KCl soluble fraction rather than into the kappa carrageenan.

Nitrate and molybdate also had relatively little effect on net ^{14}C incorporation (Table 24).

Algal strain affected ^{35}S labelling. The T_4 gametophyte showed enhanced labelling of the KCl soluble fraction with ^{35}S when compared to both Blue 7 and White 16. In the KCl insoluble fractions the Blue 7 strain showed depressed labelling. With ^{14}C no great differences were observed.

Thus it would appear that molybdate and tungstate have little effect in vivo at the concentrations used. The action of sulphate "analogues" (other Group VI anions) may be very complex as found in many higher plant systems (39). The effects seen with various fixed nitrogen sources and strain differences suggest a growth status effect rather than direct action at the level of carrageenan synthesis. It would appear that differences in growth status are

reflected in the ratio of synthesis of KCl soluble to insoluble fractions.

5. Kappa carrageenan biosynthesis

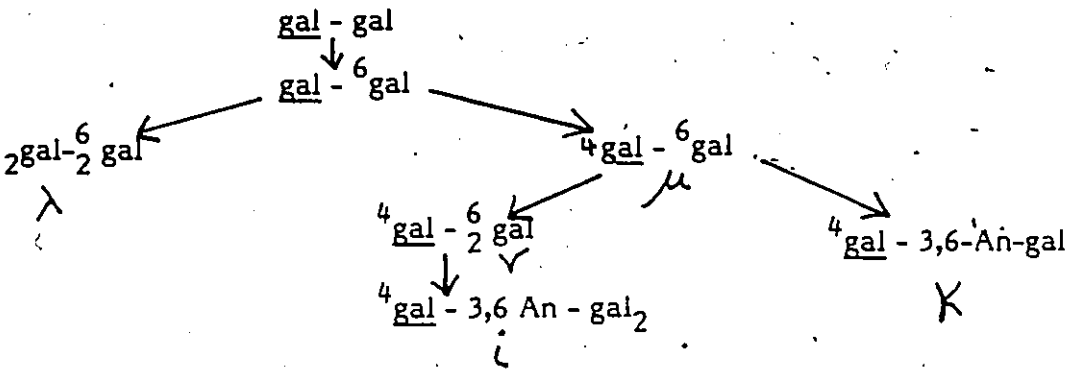
Any previously proposed pathways for carrageenan synthesis have been based primarily on relative chemical structure with presumed in vivo mimicking of in vitro chemical reactions rather than actual in vivo work. (An example of the latter is 3,6 anhydro galactose formation from 4-linked C6 sulphated galactose unit with a free hydroxyl at C3 (68)). Rees (68) originally proposed a kappa precursor unit similar to a lambda type of unit. The early work of Rees and coworkers did not distinguish between a C6 sulphate alone or a 2,6 disulphate on the 4-linked unit except by susceptibility to periodate oxidation prior to alkaline borohydride treatment in vitro (70). Both types of units were found in varying degree. Lawson and Rees (35) reported enzyme activity in Gigartina stellata which increased 3,6 anhydro-galactose content with elimination of 6 sulphate. The substrate used was 1 M KCl soluble carrageenan from G. stellata. The authors reported that they did not know if the actual substrate were galactose-6-sulphate or galactose 2,6 disulphate or both.

Yaphe (82) proposed a possible pathway or rather pathways for carrageenan synthesis (Fig. 14). In this scheme an alternating α 1,3, β 1,4 linked galactan would be sulphated on C6 of the 4-linked unit. Lambda carrageenan could therefore arise by C2 sulphation of both units. Kappa carrageenan would arise via "μ" carrageenan (formed by C4 sulphation of the 3-linked unit) upon elimination of C6 sulphate with concomitant introduction of the 3,6 anhydro ring.

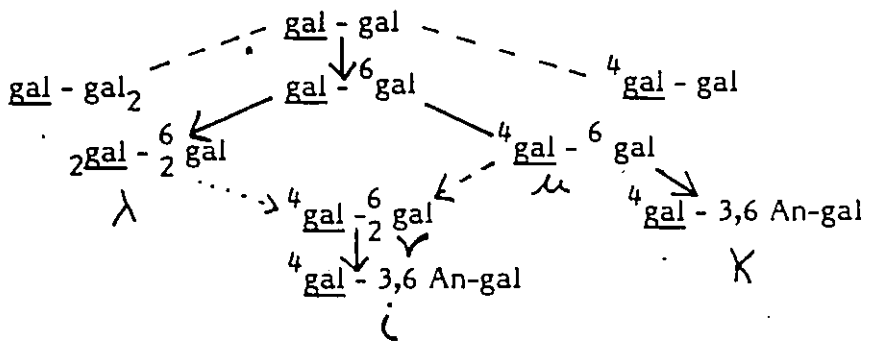
Fig. 14 Proposed pathways of synthesis of carrageenans from the literature. gal represents the 3-linked unit and gal the 4-linked unit. The subscripts and superscripts refer to the position of sulphate residues. 3,6 An refers to the presence of the 3,6 anhydro ring.

Fig. 14 Previously Proposed Pathways of Carrageenan Synthesis

1. Yaphe (82)



2. Craigie and Wong (17)



Iota carrageenan would be produced from "μ" by C2 sulphation of the 4-linked unit forming nu carageenan followed by 3,6 anhydro ring formation in this unit. Species differences were postulated to occur in certain of these steps.

In Furcellaria fastigiata (82) there is incomplete C4 sulphation in the 3-linked unit in KCl insoluble polysaccharide. In Eucheuma spinosum C2 sulphation of the 3,6 anhydro galactose residue is the rule (i.e. iota carrageenan predominates) (82).

The scheme presented by Craigie and Wong (17) allowed for C2 sulphation of the 4-linked unit and C4 sulphation of the 3-linked unit of an α 1,3 and β 1,4 linked galactan "precursor". Wong and Craigie isolated the sulphohydrolase enzyme activity which introduced the 3,6 anhydro ring with equimolar release of sulphate (80). Previous attempts by our laboratory and by Mayo-Harding (45) to isolate such activity from C. crispus had been unsuccessful.

One of the great problems in assaying such activity was the lack of knowledge of the actual substrate. Mayo Harding (45) used a lambda carrageenan (0.3M KCl soluble carrageenan from tetrasporic plants). Wong and Craigie used a "μ" substrate (80). This carrageenan was not however a chemically pure "μ" carrageenan but a 1M KCl soluble carrageenan fraction. Wong and Craigie found differences in activity with different preparations. The highest activity was found with the "enzyme resistant fraction" (ERF) (the kappa carrageenan fraction resistant to prolonged hydrolysis by kappa carrageenase from Pseudomonas carrageenovora (78, 79)).

Enzyme activity was found in the diploid plant but only using the "enzyme resistant fraction" as substrate (with their "mu" carrageenan only a trace of activity was detectable.) It is unfortunate that this system was not tested to confirm inhibition by lambda carrageenan as had been observed with enzyme activity in the haploid plant and with Gigartina stellata (35).

The "enzyme resistant fraction" contained less anhydro galactose and more sulphate than kappa carrageenan and both untreated and alkali treated ERF had greater electrophoretic mobility in starch gel electrophoresis (78, 79). After alkaline borohydride treatment ERF was partially degraded by kappa carrageenase and showed infrared bands at 930 cm^{-1} (3,6 anhydro galactose) and 805 cm^{-1} (3,6 anhydro galactose-2-sulphate). Weigl and Yaphe thus found the ERF to have many qualities of the postulated nu carrageenan. Recently Yaphe detected iota fragments in ERF by ^{13}C-nmr (personal communication to Dr. E. McCandless). They concluded that this fraction represented a biosynthetically unfinished polysaccharide (79). Wong and Craigie (80) agreed with this conclusion.

Thus the ambiguity regarding the actual precursor of kappa carrageenan had continued up to the present. The radiotracer experiments and fractionation procedures described herein provided evidence that the precursor molecules of kappa carrageenan were found predominantly in the 0.3 M KCl soluble carrageenan fraction from C. crispus but that these precursor molecules may be closer to iota type and/or nu type carrageenans in chemical structure and other properties than to mu carrageenan.

If mu carrageenan or a similarly sulphated carrageenan were the direct precursor of kappa carrageenan and if such a polysaccharide constituted the majority of the KCl soluble fraction then one might expect a 2-fold higher initial incorporation of $^{35}SO_4^{2-}$ into soluble versus insoluble carrageenan (assuming synthesis rates were similar and the smaller soluble fraction had not reached a maximum level of radioactive precursors). This ratio should decrease with time if kappa carrageenan is the final product. The ratio would also decrease if radioactive precursors ceased to enter the soluble fraction but synthesis of kappa carrageenan continued. Actual values deviated greatly from this value. Where active carrageenan synthesis continued the actual ratios were higher (soluble/insoluble: 3 to 9).

With ^{14}C the ratio under the initial conditions described would be 1. Actual values again deviated from this value. With shortened labelling times the ratio was greatly exceeded (1.6 to 180). With prolonged labelling, ratios were lower perhaps reflecting shut-down of carrageenan synthesis commencing with the soluble fraction. In pulse-chase experiments the ratios for both S and C decreased with prolonged chase periods.

As illustrated with the preliminary pulse-chase experiments (Tables 11, 14) specific activity values provided more definitive data regarding biosynthesis. In order to compare specific activity values of carrageenan fractions, however, the difference in amounts of these fractions must be considered. In this system the amount of each fraction must be equated with yield. One may then consider the various relationships which could exist between

any two such fractions.

If two fractions were labelled at an equal rate, the ratio of specific activities of the smaller to the larger would be expected to be the reciprocal of the amount of smaller/larger fraction providing certain conditions applied: viz only if there were no exit of label from the fractions and saturation with radioactive components had not been reached; the relative amounts of a particular label on a molar basis must also be considered. For example if one fraction were four times the size of the other, then the initial ratio of specific activities would be four. One may designate this ratio as the "theoretical ratio" (T.R.). Therefore T.R.= yield of the larger carrageenan fraction/yield of smaller carrageenan fraction and should then = specific activity of the smaller fraction / specific activity of the larger if the above conditions were met.

The situation in C. crispus is more complex. Precursors in the smaller KCl soluble fraction are synthesized first and then converted to kappa carrageenan. Thus the "experimental ratio" (E.R.) (the actual ratio of specific activities KCl soluble/insoluble) should be higher initially and tend to decline. In C. crispus the yield of a given fraction is quite constant over the times tested. Shifts in synthesis of KCl soluble and insoluble carrageenan fractions would be reflected in shifts in ER compared to TR.

When the E.R.'s were compared with the T.R.'s the former values over short labelling times with ^{35}S tended to exceed, to a greater or lesser degree, the latter (Results Tables 12, 13, 18). Over longer labelling times the ratio tended to drop as expected for a precursor-product relationship with the

product having little or no exit of label. The drop in ratio with ^{35}S reflected lower counts in the soluble fraction with stable or increased counts in the insoluble fraction (Tables 9, 12, 13) perhaps reflecting a shut-down of carrageenan synthesis at or before the KCl soluble fraction.

With ^{14}C the initial E.R.'s were also greater than the T.R.'s over the shorter labelling times (Table 12). Over the 48 to 96 hr period, however, with other seaweed samples virtually all ratios (^{35}S and ^{14}C) were < 1 (Table 14).

It would appear that "growth status" enters the type of analysis described above. The various experiments are internally consistent in actual ratios. It would appear that observation of the trend in ratio, i.e. increase or decrease, would be the decisive factor.

Pulse-chase experiments could help to resolve the dilemma by diminishing the input of radioactive precursors into the soluble fraction (such entry blurs changes in specific activity associated with exit from the soluble to the insoluble fraction).

In order to resolve more closely the precursor soluble fraction the 0.3M KCl was subfractionated into 2 components with 3M KCl precipitation. It is evident from the infrared scans in Figs. 11 & 12 that the two fractions have distinct predominant features (although overlap of types is not excluded).

The most significant difference between the 3M KCl soluble and

insoluble fractions lies in the critical 800-850 cm^{-1} area where different sulphate patterns are reflected in different peaks. The 3M KCl soluble fraction shows a broad absorbance from 800 -850 cm^{-1}. One problem in interpretation is the fact that the 840-850 cm^{-1} peak has been assigned to secondary axial sulphate residues (such as the C4 sulphate on the 3-linked galactose unit). However the C2 sulphate on the 3,6 anhydro galactose unit is also a secondary axial sulphate and should absorb in the same range (75). Since a 3,6 anhydro galactose peak is present in this fraction, resolution of the contribution of these two sulphates to the 840-850 cm^{-1} absorbance is difficult.

One method of resolving some of the ambiguity is to compare the 805 cm^{-1} peak which has been shown to be proportional to the 3,6 anhydro galactose content with the 3,6 anhydro galactose peak at 930 cm^{-1}. Table 31 shows a comparison of the absorbance at 805/930 cm^{-1} in various fractions. A distinct trend is evident showing decreasing ratios of C2 sulphate on the 3,6 anhydro galactose. In the sporophyte there is no distinct peak at 930 cm^{-1} as expected. In the gametophyte these ratios suggest an ever decreasing "iota-like" character with the 3M KCl soluble $>$ 3M KCl insoluble $>$ 0.3M KCl insoluble (unfractionated 0.3M KCl soluble carrageenan is intermediate in this property as predicted). The presence of C2 sulphate on the 3,6-anhydro-galactose unit of carrageenans from gametophytes of several species of red algae as an obligatory component for molecules destined for k-carrageenan biosynthesis (0.3M KCl soluble fractions) has been shown recently by DiNinno and McCandless (19).

Another very different feature of the infrared scan of the 3M KCl

Table 31. The Ratio of Absorbency at 805/930 cm^{-1} in Infrared Spectra of Various Carrageenan Fractions from C. crispus (means ± variation from the mean)

Fraction	Ratio of Absorbance	Number of Samples
0.3M KCl soluble (sporophyte)	$2.30^{+0.60}_{-0.61}$	3
3M KCl soluble (gametophyte)	1.03 ± 0.03	2
0.3M KCl soluble (gametophyte)	$0.813^{+0.10}_{-0.12}$	4
3M KCl insoluble (gametophyte)	$0.536^{+0.02}_{-0.02}$	3
0.3M KCl insoluble (gametophyte)	$0.284^{+0.07}_{-0.17}$	4

soluble fraction is the very extensive band from 970-1110 cm^{-1}. Strong absorption at the lower wave numbers is seen also in lambda carrageenan (Fig. 6). Also the major sulphate peak of the 3M KCl fraction is at 835 cm^{-1} versus 842 - 845 cm^{-1} found with other gametophytic carrageenan fractions. This latter observation suggests the possibility of a relatively low C4 sulphation in the 3-linked unit compared to the insoluble fractions.

In pulse-chase experiments one would expect a sequence of labelling: early precursor(s) $\longrightarrow$ immediate precursor(s) $\longrightarrow$ product. In C. crispus gametophyte the sequence would be: active sulphate + galactose $\longrightarrow$ kappa precursor $\longrightarrow$ kappa carrageenan. These would be found in the acetone-ethanol soluble fraction, 0.3M KCl soluble (see below) fraction, and the 0.3M KCl insoluble fractions respectively. Components of the 0.3M KCl soluble carrageenan fraction conform to limitations on structure necessary for a kappa carrageenan precursor. For example, kappa precursor must have the α 1,3 and β 1,4 galactan structure and must have a C6 sulphate on the 4-linked unit to allow formation of the 3,6 anhydro ring.

In comparing specific activity values of the two pulse-chase experiments consideration of the above sequence of synthesis is very important. The two experiments are qualitatively as well as quantitatively different because of the differences in incorporation by T4 samples. The first T4 sample was grown at high density while the second was cultured under "normal" culture conditions (B. Ives, personal communication). Although sulphate uptake from the medium was roughly equivalent in the two samples, incorporation was very

different.

In the first experiment (Table 28) both ^{35}S and ^{14}C were highest in the acetone-ethanol soluble fraction after the 2 hr chase and then both decreased. In the second experiment (Table 29) ^{35}S continued to accumulate in the acetone-ethanol fraction during the chase and ^{14}C labelling was quite constant during the chase. Thus in the first experiment the amount of labelled early precursor (active sulphate or galactose) entering the kappa precursor pool (0.3M KCl soluble fraction) was dropping during the prolonged chase. In the second experiment labelled early precursors were entering the kappa precursor pool at an increasing (^{35}S) or constant (^{14}C) rate during the chase. In the second experiment then conversion of kappa precursor to kappa carrageenan would not show as great a shift in specific activity because radioactive label would be entering the kappa precursor pool from the acetone-ethanol fraction at an undiminished rate. Such blurring of shifts in labelling was most evident with ^{35}S labelling of the 0.3M KCl soluble carrageenan fraction (Tables 28 and 29).

However, specific activity values of the 0.3M KCl soluble fractions in pulse-chase experiments were much greater than those of the 0.3M KCl insoluble fractions (Tables 28 and 29). When the ratios of specific activities are considered the ER in the second experiment would be expected to decrease less than that in the first experiment over a prolonged chase because of the blurring of labelling discussed above. Tables 32 and 33 show the comparison of ER and TR in the first (Table 28) and second (Table 29) pulse-chase experiments (ER/TR).

Table 32 Comparison of Experimental Ratio* and Theoretical Ratio in a Pulse-Chase Experiment with T_4 Gametophyte

A. Pulse with ^{35}S

	Time				
	0	1.5 hr_p^+	2 hr_c^+	24 hr_c	48 hr_c
0.3M sol/[x] 0.3M insol	0.56	5.14	4.94	6.44	1.93
3M insol/ 0.3M insol	231	273	922	199	566

B. Pulse with ^{14}C

	Time				
	0	1.5 hr_p	2 hr_c	24 hr_c	48 hr_c
0.3M sol/ 0.3M insol	11.8	186	32.2	7.90	4.36
3M insol/ 0.3M insol	9.44	1490	245	31.7	20.8

* This ratio for ^{35}S would be 2 and for ^{14}C would be 1 initially for equivalent synthesis (The ratio would decrease with the chase)

+ Subscript p refers to pulse time and subscript c refers to chase time.

x Abbreviation sol refers to KCl soluble and insol to KCl insoluble fractions at the given concentrations.

Table 33 Comparison of Experimental Ratios* in a Pulse-Chase Experiment with T_4 Gametophyte

A. Pulse with ^{35}S

	Time				
	0	1.5 hr_p^x	2 hr_c^x	48 hr_c	96 hr_c
0.3M sol/$^+$	1.4	3.5	4.3	2.2	0.72
0.3M insol	$\pm$1.1	$\pm$1.8	$\pm$3.1	$\pm$1.2	$\pm$0.26
3M sol/	1.1	2.2	1.5	1.2	0.35
0.3M insol	$\pm$0.9	$\pm$1.1	$\pm$0.9	$\pm$0.4	$\pm$0.1
3M insol/	0.10	0.77	0.20	0.55	0.21
0.3M insol	$\pm$0.06	$\pm$0.7	$\pm$0.10	$\pm$0.50	$\pm$0.04

B. Pulse with ^{14}C

	Time				
	0	1.5 hr_p	2 hr_c	48 hr_c	96 hr_c
0.3m sol/	0.49	1.04	2.7		0.92
0.3M insol	$\pm$0.06	$\pm$0.20	$\pm$1.2		$\pm$0.63
3M sol/	0.47	0.83	1.2		0.51
0.3M insol	$\pm$0.14	$\pm$0.20	$\pm$0.6		$\pm$0.2
3M insol/	0.03	0.10	0.40		0.35
0.3M insol	$\pm$0.03	$\pm$0.03	$\pm$0.31		$\pm$0.30

*Theoretical ratio for ^{35}S would be 2 and for ^{14}C would be 1 initially for equivalent synthesis (The ratio would decrease with the chase)

x Subscript p refers to pulse time and subscript c refers to chase time.

+ Abbreviations sol refers to KCl soluble and insol to KCl insoluble fractions at the given concentrations.

The 0.3M KCl soluble fraction in the first experiment (Table 32) and the 0.3M and 3M KCl soluble fractions in the second experiment (Table 33) show ER/TR values equal to or greater than expected. These ratios were thus consistent with sequential labelling of kappa precursor and then kappa carrageenan.

The 0.3M KCl soluble - 3M KCl insoluble (intermediate)fraction appeared to be the most "unpredictable" fraction in these studies. In the first experiment ER/TR ratios greatly exceeded expectation (Table 32) but in the second experiment the ratios were lower (Table 33). This latter result could indicate a shift toward 0.3M KCl insoluble carrageenan or alternatively a very slow conversion of 3M KCl soluble to 3M KCl insoluble (intermediate fraction) with a very rapid conversion of this to kappa carrageenan.

Actual yields of 3M KCl insoluble (intermediate) fraction were the most variable as were specific activity values. In the reproducibility experiment with T4 this fraction was the only one showing overlap of specific activity values at the time points tested. These results suggest that the 3M KCl subfractionation was a somewhat artificial one in respect to chemical structure. Pernas et al (58) referred to KCl solubility cut-off in the following way: "The fact that correlation between solubility and 3,6 anhydro galactose content is different for the three samples investigated demonstrates that solubility is not controlled solely by the 3,6 anhydrogalactose content, and it seems reasonable to associate this deviation with variation in the sulphate content or distribution". The data suggest that the 3M KCl insoluble fraction could represent a transition

stage. The possibility that C_4 sulphation of the 3-linked unit might be incomplete or C_2 desulphation of the 4-linked unit had begun were not ruled out by infrared analysis.

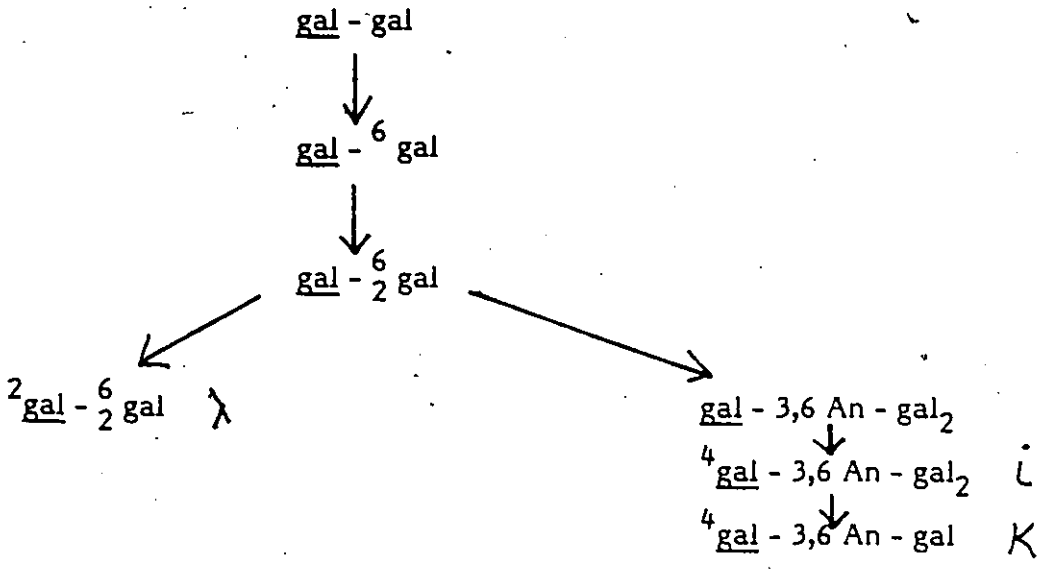
These pulse-chase experiments do confirm the labelling pattern expected on the basis of a sequence: early precursor $\longrightarrow$ immediate precursor $\rightarrow$product. Prolonged labelling studies and preliminary "pulse-chase" studies also support the sequence. Chemical evidence limits the possible choice of a kappa precursor. This evidence coupled with immunological and infrared analyses support the possible biosynthetic pathway shown in Fig 15.

The relationships between carrageenan fractions are straightforward. In the synthesis of kappa carrageenan it would appear that an "extra" sulphation step takes place. The C2 sulphation of the 4-linked unit however may positively affect the stability of the very labile 3,6 anhydro ring (68). Also the advantage to the plant of a common precursor for lambda and kappa carrageenan ($\underline{\text{gal}}$-$^{6}_{2}$ gal) provides the "switch" mechanism for the rapid changes in polysaccharide type with ploidy change seen with the antibody techniques (28). Also by eliminating the requirement for other interconversion enzymes the "extra" sulphation step may be energy conserving overall. Gel strength and conformation may also be factors as iota and kappa carrageenans differ in gel strength in the presence of certain ions.

Basically three possibilities exist at the initial stages of synthesis. First there could be synthesis of a large non-sulphated α 1,3 and β 1,4 galactans

Fig. 15 Proposed pathway of synthesis of carrageenans from this thesis. gal represents the 3-linked unit and gal the 4-linked unit. The subscripts and superscripts refer to the position of sulphate residues. 3,6 An refers to the presence of the 3,6 anhydro ring.

Fig. 15 Possible Biosynthetic Pathway for Carrageenans



which would be sulphated at the polymer level. Second there could be small α 1,3 and β 1,4 galactans which would be sulphated and then linked together to form a large polysaccharide or such units could be added to a pre-existing polymer. The third possibility is that single galactose residues could be sulphated and then linked together to form a polymer or added separately to a pre-existing polymer.

The first possibility is ruled out by the pulse-chase experiments reported here because of closeness in time of ^{35}S and ^{14}C labelling of carrageenan fractions (Tables 27 and 28). Also previous labelling and fractionation studies (3,4) showed no evidence of a large non-sulphated polygalactan. Floridean starch, a glucose polymer, is found to be the major non-sulphated polysaccharide. (4). Similarly no evidence has been found of any significant quantity of sulphated galactose units (3,4).

Thus the second possibility remains the most attractive. Bidwell (4) had found an unknown substance in the 50% alcohol soluble fraction which yielded only galactose upon hydolysis. The site of sulphation of such a precursor is also a question mark in the early stages of carrageenan synthesis. There is evidence in the brown algae (23) and in a unicellular red alga (67) that some sulphation takes place in the Golgi apparatus. Evans et al have reported autoradiographic evidence of sulphation in the Golgi-rich perinuclear regions of both Pelvetia and Laminaria spp. (the latter having specialized secretory cells) (23). Similarly Ramus (62) and Ramus and Robins (67) presented electron microscopic evidence for Golgi involvement in synthesis of sulphated

polysaccharide in the unicellular Porphyridium aerugineum. They found vescicles, containing fibrillar material, which presumably migrated to the cell surface where the contents were released.

It is interesting to note that Wong and Craigie (80) found 65% of the sulphohydrolase activity in C. crispus associated with the particulate fraction. Whether this is cell wall or membrane associated or Golgi associated must be resolved by subfractionation techniques. This activity however must necessarily follow sulphation since introduction of the 3,6 anhydro ring requires elimination of a strong anion at C6 (70).

Certain sulphation and/or desulphation reactions appear to occur in highly polymerized polysaccharides. Certainly it would appear that this introduction of the 3,6 anhydro ring with the elimination of C6 sulphate takes place at the polymer level (3,6 anhydro galactose content increases and sulphate tends to decrease from the 0.3M KCl soluble to insoluble carrageenan (47)). Certainly the transition from iota-like to kappa carrageenan reported here suggests C2 sulphohydrolase activity at the large polymer level. The ambiguity in infrared scan analysis in the 840-850 cm^{-1} range and the properties of the 3M KCl soluble fraction also suggest that C4 sulphation could proceed at a later stage. Certainly the very rapid transition in antibody specificity seen with fluorescent antibody staining implies modification of a pre-existing common precursor rather than total de novo synthesis (28). In the scheme suggested this would be the small galactan or an α 1,3 and β 1,4 galactose-2,6-disulphate polymer.

CONCLUSIONS

As a result of the development here of experimental conditions suitable to obtain high specific activity labelling of carrageenan fractions with ^{35}S-sulphate and ^{14}C-bicarbonate, carrageenan biosynthesis could be studied in detail not previously possible. Several conclusions can be made from this work.

1. Sulphate uptake is related to growth status rather than life cycle stage and is not directly related to internal requirement as reflected by incorporation into carrageenan fractions.

2. ^{14}C-bicarbonate uptake also appears not to be significantly related to life cycle stage.

3. Lambda carrageenan is synthesized more rapidly than kappa carrageenan but incorporation into total carrageenans (KCl soluble and insoluble fractions) is similar in sporophyte and gametophyte.

4. Sulphate level in the medium affects both sulphate uptake and incorporation into carrageenan fractions but there is not a direct relationship.

5. Sulphate analogues molybdate and tungstate have no significant effect on sulphate uptake or incorporation into carrageenan fractions

under the conditions used.

6. Light intensity affects sulphate uptake and incorporation in a complex manner and has differential effects on non-cultured wild-type, cultured wild-type, and non-cultured T_4 gametophytes. Dark conditions virtually eliminate ^{14}C-bicarbonate incorporation into carrageenan fractions.

7. Different fixed nitrogen sources affect carrageenan synthesis in different ways.

8. Certain strains of C. crispus show differences in ^{35}S-sulphate uptake and incorporation compared to T_4 gametophyte but no significant changes are seen among strains with ^{14}C-bicarbonate.

9. The precursor to kappa carrageenan is present in the KCl soluble fraction in the gametophyte. On the basis of radiolabelling, immunochemical, and infrared data the precursor is more iota and/or nu carrageenanlike than the previously proposed precursor, mu carrageenan.

10. The proposed biosynthetic pathway for kappa carrageenan is consistent with all evidence so far published and reported here and is considerably less complex than previously proposed pathways.

APPENDIX A. MEDIA

1. Artificial seawater media

i. Bacto-Marine broth (Difco)

Ingredient	Amount/ l
Bacto-peptone	5 g
Bacto-yeast extract	1 g
Ferric citrate	0.1 g
Sodium chloride	19.45 g
Magnesium chloride dried	5.9 g
Sodium sulphate	3.24 g
Calcium chloride	1.8 g
Potassium chloride	0.55 g
Sodium bicarbonate	0.16 g
Potassium bromide	80 mg
Strontium chloride	34 mg
Boric acid	22 mg
Sodium solicate	4 mg
Sodium fluoride	2.4 mg
Ammonium nitrate	1.6 mg
Disodium phosphate	8 mg

ii. N-marine broth

Bacto-marine broth as above plus 10 ml of 0.05M NH_4NO_3

iii. Marine agar

Bacto-marine broth plus 18 g Difco Bacto-agar

iv. N-marine agar

Marine agar as above plus 10 ml of 0.05M NH_4NO_3

v. Artificial seawater - Novotny and Forman (56)

Ingredient	Amount
NaCl	32.3 g
$MgCl_2.6H_2O$	10.5 g
$CaCl_2.2H_2O$	1.46 g
$NaHCO_3$	0.19 g
KCl	0.66 g
glass distilled H_2O	955 ml

The pH is adjusted to 7.5.

2. Enriched seawater media

i. B5(s) adapted from B5 medium (14)

A. Stock solutions

a. Micronutrients (stored frozen)

Ingredient-	mg/100 ml
$MnSO_4.H_2O$	1000
H_3BO_3	300
$ZnSO_4.7H_2O$	200
$NaMoO_4.2H_2O$	25
$CuSO_4.5H_2O$	2.5
$CoCl_2.6H_2O$	2.5

b. Vitamins (store frozen)

Ingredient	mg/100 ml
Nicotinic acid	100
Thiamine.HCl	1000
Pyridoxine.HCl	100
m-inositol	10,000

c. Calcium chloride

$CaCl_2.2H_2O$	15 g/100 ml

d. Potassium iodide

KI	75 mg/100 ml

Store in amber bottle in refrigerator.

e. 2,4-dichlorophenoxyacetic acid (2,4-D) 2.2 mM

Dissolve 50 mg 2,4-D in 2-5 ml ethanol, heat slightly to dissolve and gradually dilute to 100 m. with water. Store in refrigerator.

f. Kinetin 1 mM

Dissolve 21.5 mg of kinetin in a small volume of 0.5 N HCl by heating slightly and tradually diluting to 100 ml with distilled water. Store in refrigerator.

B. Preparation of B5 (s)

Ingredient	Amount/ 1
$NaH_2PO_4.H_2O$	150 mg
KNO_3	2500 mg
$(NH_4)_2SO_4$	134 mg
$MgSO_4.7H_2O$	250 mg
$FeCl_3.6H_2O$	31 mg
Na_2EDTA	33 mg
Sucrose	20 g
$CaCl_2$ stock	1.0 ml
Micronutrients stock	1.0 ml
KI stock	1.0 ml
Vitamins stock	1.0 ml
Seawater	1000 ml

The pH is adjusted to 7.6

ii. 1-B5(s)

1-B5(s) is B5(s) above with the addition of 2.0 m. of 2,4-D stock solution.

iii. Enriched seawater medium-SWM 3 (50) as described by Harding (45).

A. Stock solutions

a. Vitamins

Ingredient	Amount/2 ml
Thiamine.HCl	0.5 mg
Ca pantothenate	0.1 mg
Nicotinic acid	0.1 mg
p-amino benzoic acid	10 µg
Biotin	1.0 µg
Inositol	5 mg
Folic acid	2 µg
Thymine	3 µg
B_{12}	1.0 µg

b. Trace metals

Ingredient	Amount/2 ml
H_3BO_3	12.4 mg
$MnCl_2$	1.39 mg
$ZnCl_2$	0.109 mg
$CoCl_2$	4.759 µg
$CuCl_2$	0.02689 µg

c. Soil extract

1. Add 2 l tap water to 1 kg find garden soil (commercially available potting soil was used) and mix well.

2. Autoclave at 15 psi 20 min in a foil covered beaker.

3. Pour slurry into a tall cylinder and allow to stand overnight.

4. Filter through a Celite pade and a double layer of Whatman #1 filter paper on a large Buchner funnel (The Celite was omitted).

5. Refilter if necessary through a 0.45u membrane filter (This step was omitted)

6. Dispense in plastic vials and freeze for future use. Use 50 ml per litre of medium (Soil extract was refiltered before use)

d. Na_2EDTA - $FeCl_3$ (adapted from Von Stosch)

1. Dissolve 5 g $Na_2EDTA.2H_2O$ in 200 ml distilled water

2. Dissolve 300 mg anhydrous $FeCl_3$ or 500 mg $FeCl_3.6H_2O$ in a separate beaker in about 20 ml distilled water

3. Mix $FeCl_3$ solution with Na_2EDTA solution and dilute to 800 ml with distilled water.

4. Adjust pH to 7.5 with NaOH

5. Dilute to 1000 ml. Use 1.0 ml per liter medium..

B. Preparation of medium

Ingredient	Amount/ 1
$NaNO_3$	0.17 g
$NaHPO_4.2H_2O$	0.0156 g
$EDTA.FeCl_3$	1 ml stock
Trace metals	2 ml stock
Vitamins	2 ml stock
Soil extract	50 ml stock
Tris buffer	500 mg
GeO_2	2 ml of a saturated solution
$NaHCO_3$[a]	5 ml of 400 mM

[a]Added after autoclaving.

3. Protoplast media

1. 1B5(s) + 2 mg /1 kinetin
2. Seawater + 1% glucose + 2mg/1 2,4-D

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