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MICROBIAL DEGRADATION OF GUM KARAYA*

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ABSTRACT

Gum karaya (*Sterculia urens* gum) was degraded by a fungal isolate, a *Cephalosporium sp.* The patterns of carbohydrate utilization and enzyme secretion by the organism growing on the purified polysaccharide were determined, together with the ratio of neutral sugars present in the gum. *Cephalosporium* growth-studies indicate that the gum contains at least three different types of chains. One chain (50% of the total polysaccharide) is postulated to contain repeating units of four galacturonic acid residues containing β-D-galactose branches and having an L-rhamnose residue at the reducing end of the unit. A second chain (17% of the polysaccharide) contained 50% of galacturonic acid, 40% of rhamnose, and 10% of galactose by weight and is postulated to contain an oligorhamnan chain, containing D-galacturonic acid branch-residues, and interrupted occasionally by a D-galactose residue. D-Glucuronic acid is apparently confined to a third type of chain, comprising 33% of the polysaccharide.

INTRODUCTION

Exudate gums are complex polysaccharides that are produced by plants spontaneously or in response to adverse conditions or mechanical injury[1]. Smith and Montgomery[2] have postulated that such complex polysaccharides, the synthesis of which must involve a highly organized, complex system of enzymes, are probably immune to the degradative enzymes of most bacteria and fungi.

Beauquesne[3] found that *Sterculia tomentosa* gum was resistant to attack by the hepato-pancreatic enzyme complex of the snail (*Helix sp.*), by *Penicillium erlichii*, and by several species of *Clostridium*. Aspinall and Baillie[4] found that gum tragacanth (*Astralgus gummifer*) is not attacked to any appreciable extent by commercial pectinase and hemicellulase preparations. However, after controlled acid hydrolysis, tragacanthic acid was attacked by these enzymes.

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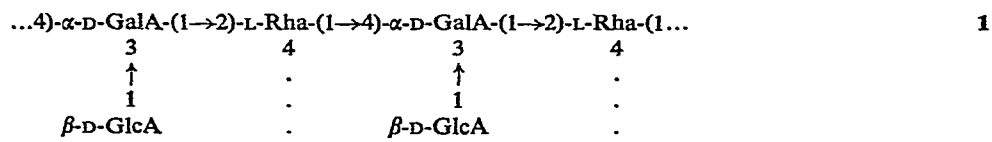
Nagel[5] attempted to degrade gum karaya (*Sterculia urens*) using microorganisms and a commercial glycosidase preparation (Pectinol 10-M), and found that the gum is not attacked to a measurable extent by either. The microbial inocula were enrichment cultures from sewage, soil, river water, and other sources, as well as many fungal contaminants (from the air) on other polysaccharides in the laboratory.

Evidence for the structure of gum karaya is still fragmentary. Kubal[6], and Kubal and Gralen[7] have reported the gum to have a molecular weight of approximately 9,500,000, and have postulated that the tertiary structure in colloidal suspension behaves as "matted coils" having the solvent immobilized within the coils of the molecules. They also obtained evidence indicating that the molecule is extremely asymmetric, having the approximate dimensions of 86 by 2750 Å which, if true, indicates that the molecule is very tightly packed.

The evidence thus far for the primary structure of the gum has been reported by Aspinall and various coworkers[8–10]. Aspinall and Nasir-Ud-Din[8] have shown that the gum contains galactose, galacturonic acid, rhamnose, and glucuronic acid, and by partial acid hydrolysis they have isolated and determined the structures of several acidic oligosaccharides. In two recent papers, Aspinall and Sanderson[9,10] have reported the isolation and characterization of a variety of new acidic oligosaccharides, obtained from gum karaya and from the carboxyl-reduced gum by partial acid hydrolysis and acetolysis. After considering all of their evidence, Aspinall and Sanderson[10] proposed the following structures for the polysaccharide chain:



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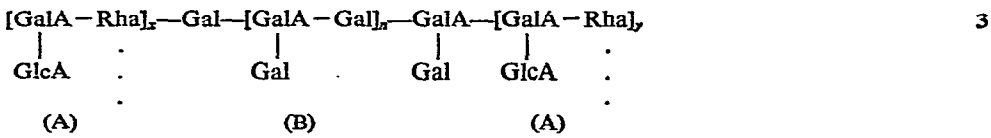
They were unable to find any evidence as to which residue or residues are (1→4)-linked to the L-rhamnose residues. Chain **2** was postulated from evidence obtained by acetolysis of the carboxyl-reduced gum, which indicated that the 2-linked galactose residue is a branch unit and not a repeating component in the main chain.

Aspinall and Sanderson[10] were convinced, from to the homogeneity shown by Aspinall and Nasir-Ud-Din[8] for the gum, that the two chains **1** and **2** are present as alternating blocks in the same chain, and they indicated one possible way in which these may be linked:



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No further evidence thus far for the structure of gum karaya, other than oligosaccharide analysis, has appeared in the literature. As far as we are aware, selective microbial or enzymic degradation of acidic exudate gums has not been attempted. The findings of Aspinall and Baillie[4] have already been mentioned.

We now report structural information concerning gum karaya derived from microbial degradation of that gum.

RESULTS

The upper graph in Fig. 1 shows the pattern of utilization of the purified polysaccharide by *Cephalosporium sp.*; the lower one shows the sequence of enzyme secretion. A very large peak of D-galacturonanase activity appeared at 12 h and then rapidly declined until it was only about one seventh of its original maximum. At 24 h of growth, a very small amount of α-L-rhamnosidase activity appeared, and then disappeared just as quickly. This rhamnosidase attacked methyl α-L-rhamnoside as well as *p*-nitrophenyl α-L-rhamnoside, and hence it appears not to have been strictly an aryl glycosidase. At 36 and 130 h, two step-like increases occurred in D-galacturonanase activity, which remained constant thereafter. At 155 h, β-D-galactosidase activity began to appear, and reached a plateau at ~200 h.

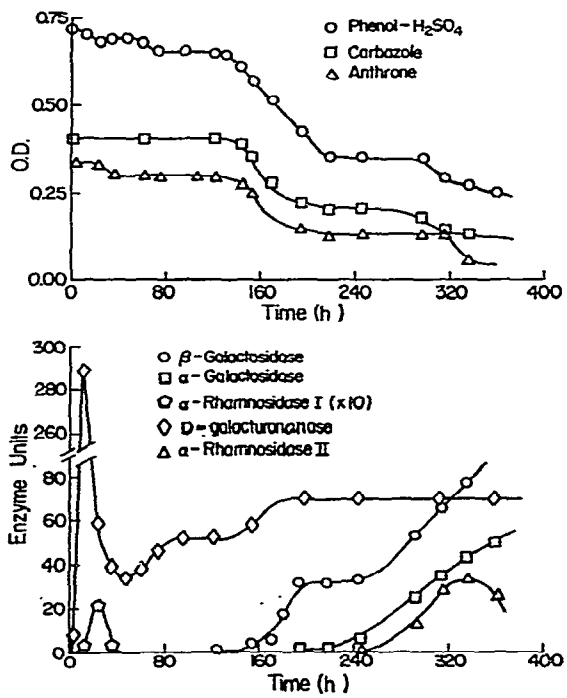


Fig. 1. Patterns of enzyme secretion and carbohydrate utilization by *Cephalosporium sp.* growing on purified gum karaya.

Up to the point at which the third rise in D-galacturonanase activity occurred, (130 h), the only indication of carbohydrate utilization was a small decrease in neutral sugar (*ca.* 5% decrease in total carbohydrate) at 24–36 h. At about 140 h, the total carbohydrate (both neutral sugar and uronic acid) began to decline rapidly. Between 140 and 220 h of growth, *ca.* 50% by weight of the total polysaccharide disappeared, corresponding to decreases in uronic acid and neutral sugar of 50% each (neutral sugar determined by difference).

No further disappearance of carbohydrate occurred until *ca.* 280 h. In the interval between the end of the initial decrease of carbohydrate and the beginning of the second decrease, *p*-nitrophenyl β-D-galactosidase activity, which leveled off at 200 h, began to rise again at a linear rate. At about the same time, *p*-nitrophenyl α-L-rhamnosidase activity began to appear, reached a maximum at *ca.* 340 h, and then began to decline. At *ca.* 210 h, *p*-nitrophenyl α-D-galactosidase activity appeared and increased at a linear rate, and began to level off at 360 h of growth.

At 280 h, a second disappearance of carbohydrate began, and this appeared to be entirely uronic acid; it was followed at 315 h by a decrease in neutral sugar. At 360 h of growth, 67% (by weight) of the total carbohydrate had been utilized. A corresponding decrease in uronic anhydride (67%) had also occurred by this time, indicating that a like proportion of neutral sugar had been used.

The ratios of rhamnose to galactose, at selected intervals during the growth of *Cephalosporium sp.* on the polysaccharide, are shown in Table I. This ratio was also determined on the deacetylated crude gum before hydrolysis at pH 2.5. A number of points are evident from Table I. First, at pH 2.5, hydrolysis of the deacetylated crude gum resulted in a greater loss of galactose than rhamnose. This indicates that most of the rhamnose is inaccessible to mild acid hydrolysis and is therefore probably in the main chain of the gum, whereas more of the galactose residues exist as branches. This observation supports the evidence of Aspinall and Nasir-Ud-Din[8].

More interesting, however, is the change that occurs in the galactose: rhamnose ratio (Table I) during the first large decrease in carbohydrate (Fig. 1) beginning at 150 h. Between 150 and 292 h 3.6 times as much galactose was removed (percentage-wise) from the gum as rhamnose. This evidence strongly supports the enzyme-secretion data in Fig. 1, showing the rapid rise in β-D-galactosidase activity during that time.

By 529 h, the organism had utilized 85% of both uronic acid and total carbohydrate (Fig. 1) and hence, by difference, 85% of the neutral sugar had been utilized also. The material remaining had a galactose:rhamnose ratio significantly higher than that at 292 h, indicating that a substantial proportion of the rhamnose remaining at 292 h had been used.

The α-D-galactosidase was not active on the alditols of melibiose or stachyose and therefore was either an aryl glycosidase or was highly aglycon- and/or linkage-specific. The enzyme was not isolated, and its effects on the polysaccharide are as yet unknown. The D-galacturonanase activity, which has been shown to be a composite

TABLE I

CHANGES IN THE GALACTOSE AND RHAMNOSE CONTENTS OF PURIFIED GUM KARAYA DURING GROWTH OF *Cephalosporium sp.*

Neutral sugar	*Deacetylated crude gum*		*130 h*		*292 h*		*529 h*	
	Rhamnose	*Galactose*	*Rhamnose*	*Galactose*	*Rhamnose*	*Galactose*	*Rhamnose*	*Galactose*
Ratio (parts/10 parts)	5.3	4.7	5.7	4.3	8.7	1.3	6.8	3.2
Sample content (μg)	30.2[a]	26.8[a]	27.9[b]	21.1[b]	21.3[c]	3.2[c]	5.0[d]	2.2[d]
Decrease (μg)			2.3[e]	5.7[e]	6.6[f]	17.9[f]	22.9[f]	18.9[f]
% Decrease			7.6	21.3	23.6	84.9	82.4	84.9

[a]Crude deacetylated gum before hydrolysis at pH 2.5 to remove bound tannin; sample content of 57 μg total neutral sugar. [b]Purified gum after hydrolysis at pH 2.5 to remove bound tannin; sample content of 49 μg total neutral sugar. Approximate molecular weight of 550,000. [c]50% of neutral sugar utilized; sample content of 24.5 μg total neutral sugar. [d]85% of neutral sugar utilized; sample content of 7.4 μg total neutral sugar. [e]Decrease from deacetylated crude gum. [f]Decrease from 130-h values, (130-h values are essentially zero-time values for the purified polysaccharide because almost no utilization had occurred at this point).

of several activities[11], caused 5.6% of hydrolysis (by reducing group assay[12]) at the point of half-maximum viscosity, indicating that total activity was somewhere between fully "exo" and fully "endo" in nature. Fully random ("endo") activities are not ruled out as components of the total D-galacturonanase activity, because they would cause only 1–2% hydrolysis. But fully "exo" components were either not present or had very low activities. Hence, it appears that the total D-galacturonanase activity either consists of a mixture of fully random enzymes plus enzymes intermediate between "endo" and "exo" in nature, or solely the latter type. At no time during the growth period was β-D-glucosiduronase detected by using either *p*-nitrophenyl β-D-glucuronic acid or oxidized cellulose [(1→4)-β-D-glucuronan] as the substrate.

A plot of reducing power during microbial growth on the gum is shown in Fig. 2. The data were obtained from a repeat of the foregoing experiment which, though differing slightly in the times of enzyme secretion, and so on, gave essentially the same pattern shown in Fig. 1. As shown in Fig. 2, reducing power began to increase within the first 30 h, when the first large peak of D-galacturonanase activity and the first α-L-rhamnosidase activity appeared. During the subsequent increases of D-galacturonanase activity, the reducing power suddenly rose sharply to a maximum and then began to decrease rapidly as utilization began (as evidenced by the decrease in phenol–sulfuric acid-reacting material)[13]. The same indication was found by using chromatography on Sephadex G-100, which showed that at 12 h the gum was considerably more fragmented than at the outset.

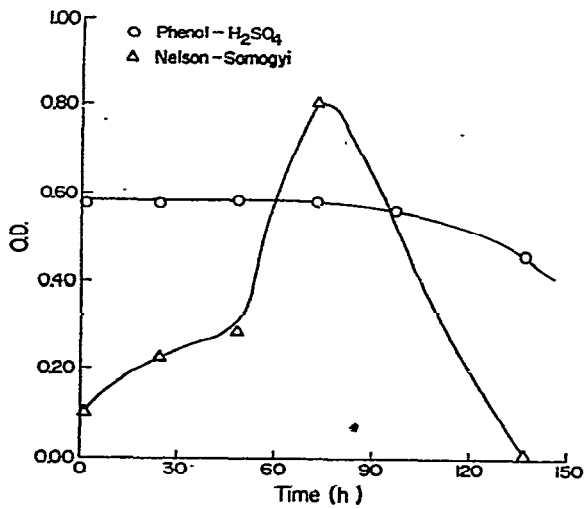


Fig. 2. Reducing groups released by *Cephalosporium sp.* during growth on purified gum karaya.

Chromatography on Sephadex G-50, as shown in Fig. 3a, revealed that up until 180 h of growth of *Cephalosporium*, the material remaining had a molecular

weight greater than 10,000*. At 130 h, the excluded peak began to decrease but no detectable material of lower molecular weight appeared. At 218 h (Fig. 3b), a shoulder of material of lower molecular weight appeared on the excluded peak and by 292 h, had become a distinct second peak. This second peak then began to disappear until it again was merely a shoulder on the excluded peak, which also decreased slightly. This result indicates that, up to 218 h, any fragment of molecular weight less than 10,000 was utilized as fast as it was split from the polysaccharide.

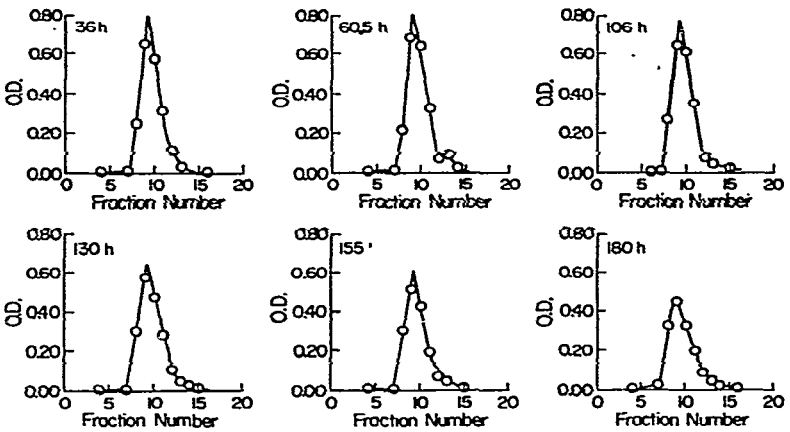


Fig. 3a.

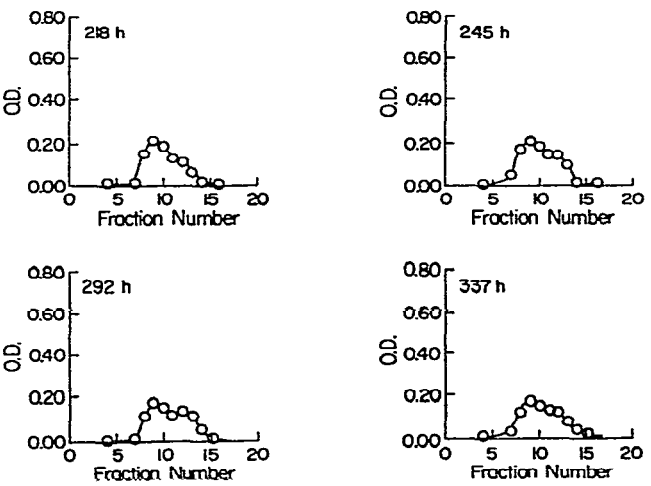


Fig. 3b.

Fig. 3a, 3b. Sephadex G-50 chromatography of samples taken at various times during growth of *Cephalosporium sp.* on purified gum karaya.

*The void-volume of the G-50 column was 60 ml (9.3 fractions of 6.5 ml). One void-volume equals the exclusion volume for material of molecular weight greater than 10,000.

The results of chromatography on Sephadex G-100 are shown* in Fig. 4. It has already been mentioned that the gum was broken down considerably after 12 h, the pattern being almost identical in a sample taken at 36 h, just after the disappearance of the first peak of α-L-rhamnosidase activity. From this observation, it would appear that little, if any, "endo" cleavage had been caused by this enzyme. The fragmentation pattern for later samples indicates that, as material of lower molecular weight (>10,000) was utilized, it was replaced by material that was being continuously cleaved from the fraction of higher molecular weight, with the result that both components disappeared until a particular chain type was left, to which the organism had to readapt in order to attack it. The material remaining from 218 h onward contained a fair amount of material of lower molecular weight, some of which was degraded only very slowly by the organism.

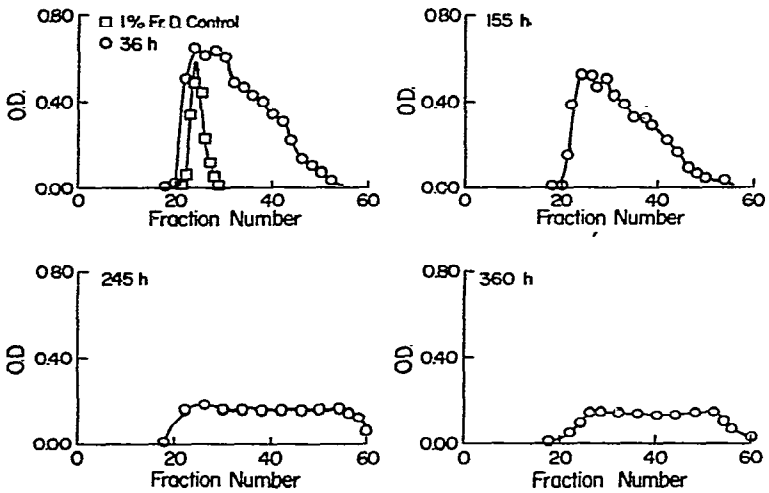


Fig. 4. Sephadex G-100 chromatography of samples taken at various times during growth of *Cephalosporium sp.* on purified gum karaya.

The three central fractions of each excluded peak shown in Figs. 3a and 3b were diluted 1/100 and analyzed by the phenol–sulfuric acid[13] and carbazole[14] assays, and the percentage of uronic acid was calculated. The results are shown in Fig. 5. Up until 106 h of growth, the uronic acid content remained relatively constant. At 155 h, just prior to the first decrease in carbazole-reacting material (Fig. 1), the uronic acid content of the fractions reached 70–75% by weight, falling back to the original level by 216 h (the point at which utilization of carbohydrate by the organism

*The void-volume of the Sephadex G-100 column was 156 ml (24 fractions of 6.5 ml). One void-volume equals the exclusion volume for material of molecular weight greater than 100,000. Dextran 10 (molecular weight, 9,200) appeared in Fraction 52 (Fig. 4) (approximately 2.2 void-volumes); the salts appeared in Fraction 74 (approximately 3.1 void-volumes).

temporarily ceased). At about 290 h, uronic acid content reached a minimum and then at 316 h rose toward the initial value as the neutral sugar was used (Fig. 1).

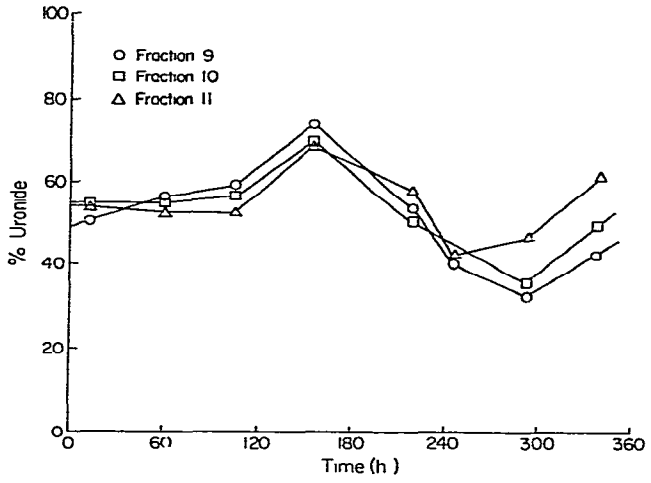


Fig. 5. Uronic acid content of Sephadex G-50-excluded fractions shown in Figs. 3a and 3b.

In order to determine the sequence of utilization of monosaccharides by *Cephalosporium sp.*, the organism was grown on a basal mineral medium containing 0.5% each of L-rhamnose, D-galactose, D-glucuronic acid, and D-galacturonic acid. *Aspergillus niger* was grown on the same medium for comparison. The disappearance of the monosaccharides, determined by gas–liquid chromatography, occurred simultaneously in the *A. niger* culture fluid. D-Glucuronic acid was used the fastest and L-rhamnose the slowest, whereas D-galactose and D-galacturonic acid were used at the same rate. *Cephalosporium sp.* also used these two monosaccharides at the same rate. D-Glucuronic acid was used more slowly and, for some reason, was not utilized completely. L-Rhamnose was not utilized until D-galactose and D-galacturonic acid were compietely used up, and, at this point, utilization of D-glucuronic acid stopped abruptly. It appears that utilization of rhamnose was repressed by either D-galactose or D-galacturonic acid or both; the system for utilization of D-glucuronic acid was apparently de-repressed by one or both of these monosaccharides.

Aspergillus niger spores were also inoculated into aliquots of the 12-, 36-, 96-, 195-, and 360-h samples taken during the degradation of the purified polysaccharide by *Cephalosporium*, and growth and utilization of the samples were compared with that for the original purified polysaccharide. In all cases, the spores germinated and hyphae appeared, but the new hyphae sporulated almost immediately and, after one week of incubation, no measurable utilization was found in any of the samples. One week should have been sufficient growth time, especially at 30°, because *A. niger* spores were able to germinate at room temperature and completely utilize a number of monosaccharides in 3 days.

DISCUSSION

The patterns for utilization of carbohydrate and secretion of enzymes by *Cephalosporium sp.* acting on the purified polysaccharide have already been described (Fig. 1). Apparently the gum was first fragmented by "endo" cleavage, as indicated by the activity in the initial large D-galacturonanase peak appearing at 12 h, because chromatography on Sephadex G-100 showed the gum to be considerably fragmented at 12 h (Fig. 4). Judging from the small initial increase in reducing power (Fig. 2), the degree of cleavage was small, as would be expected with "endo" enzymes. The first enzyme may have attacked, to some extent, the (1→4)-linkages between galacturonic acid units once removed from L-rhamnose, as shown in **4**, to yield two fragments (**5** and **6**). Exposure of α-L-rhamnoside linkages (now only one unit from the end of the chain) in **5** could then have caused induction of a small amount of α-L-rhamnosidase activity, which appeared at 24 h.

...4)-α-D-GalA-(1→2)-α-L-Rha-(1→4)-α-D-GalA-(1→4)-α-D-GalA... **4**

...4)-α-D-GalA-(1→2)-α-L-Rha-(1→4)-α-D-GalA + α-D-GalA...

5 **6**

Being an "exo"-α-L-rhamnosidase, it probably could not attack **5** and hence, no free D-galacturonic acid was released and utilized. Studies of the fractionation of the gum showed that up until 218 h of growth of *Cephalosporium*, even though the gum became highly fragmented, no material of molecular weight below 10,000 was present, again supporting "endo" cleavage.

The total data obtained during the utilization experiment indicate, as Aspinall and Sanderson[10] have suggested, that more than one type of chain structure is present in the gum molecule.

The first major disappearance of carbohydrate (60% each of uronic acid and neutral sugar) occurred while only pectic enzymes and β-D-galactosidase were present. During this time, *ca.* 85% by weight of the D-galactose present at 130 h disappeared. The L-rhamnose content decreased also by *ca.* 24%. As no L-rhamnosidase was detected by using either *p*-nitrophenyl α-L-rhamnoside or naringin, possibly a highly specific L-rhamnosidase was present at this time without its being detected. It is equally possible that L-rhamnose-containing di- or tri-saccharides were cleaved from the chain by other enzymes and then degraded immediately at the membrane or intracellular levels, in which case α-L-rhamnosidase would not have appeared in the culture fluid. As no β-D-glucosiduronase was detected, either by assays with *p*-nitrophenyl β-D-glucosiduronic acid or oxidized cellulose, the uronic

acid material that disappeared was probably all D-galacturonic acid. However, the possibility again exists that 3-*O*-(β-D-glucopyranosyluronic acid)-D-galacturonic acid was cleaved from the chain and degraded on cell membranes or by intracellular enzymes. Current studies in this laboratory with the intracellular enzymes of *Cephalosporium sp.* are directed to these questions.

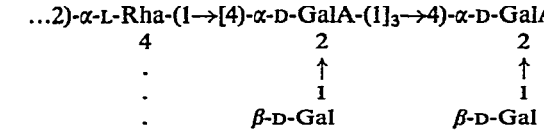
The material, which will be designated Chain I and which was utilized during the first major decrease of carbohydrate, contained *ca.* 50% by weight of uronic acid (probably D-galacturonic acid), 36% of D-galactose, and 14% of L-rhamnose, judging from the data in Fig. 1 and Table I. As some of the D-galactose present (21%) was removed during the hydrolysis of the deacetylated crude gum (Table I), the ratio of D-galactose to D-galacturonic acid in Chain I in the crude gum may have been 1:1.

In order to define the structure of this chain, one question must still be answered, namely, "How are the monosaccharide residues arranged in the chain?" The galactose residues may exist as branches, as interior units in the main chain, or a combination of both. The periodate-oxidation results of Aspinall and Nasir-Ud-Din[8] have shown that the major proportion of the galactose in the gum is in unbranched chains. These results are of no value in the present work, because unbranched, interior chain galactose residues would also be oxidized by periodate. The results of hydrolysis of the deacetylated crude gum are also inconclusive. The D-galactose removed was most probably branch-chain D-galactose, residues, but that remaining could have either been interior-chain D-galactose residues or branch units which, due to protection by the coiling of the chains, were inaccessible to hydrolysis. Enzyme studies were also inconclusive for the same reason[11].

The fact that the composite pectic-enzyme activity present at the first major decrease in carbohydrate was more "endo" than "exo" argues for the presence of oligouronic acid chains (probably trisaccharide or greater) in Chain I. This would eliminate the possibility of alternation of mono- or digalacturonic acid with neutral units in the interior chain. Also, since disappearance of neutral sugar preceded disappearance of uronic acid (Fig. 5), the uronic acid reaching a maximum at *ca.* 155 h, and since no material of molecular weight less than 10,000 appeared at this time, it is probable that most, if not all, of the galactose in Chain I was as chain-branch residues. The probability of two separate chains, one entirely of α-linked D-galacturonic acid residues and the other of β-linked D-galactose residues, is low for two reasons. First, long chains of unprotected uronic acid residues would have been degraded and utilized long before 130 h, and degradation and utilization certainly would not have depended upon the appearance of a β-D-galactosidase. By the same line of reasoning, long chains of a β-D-galactan would have been attacked by a galactanase and utilized, or by an "exo" -β-D-galactosidase after being released from the uronic acid chains by a galacturonanase. During cleavage by "exo" β-D-galactosidase, assuming that the galactan was at least 50 units long to begin with (molecular weight greater than 10,000), the chains would have become continually smaller until a molecular weight of less than 10,000 was reached, at which point the fraction of lower molecular weight would have been detectable by Sephadex G-50 chromato-

graphy (which it was not). Long chains of free galacturonan and galactan are also argued against by the fact that the gum is not attacked by many microorganisms that can attack one or both of these chain types[11].

The most likely repeating unit of Chain I, judging from the arguments presented here, is structure 7. This structure is consistent with the fact that the ratio of rhamnose to galacturonic acid in Chain I was *ca.* 1:4,



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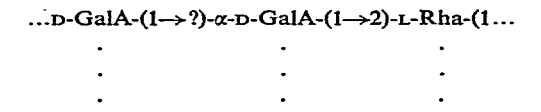
as already pointed out. It is also consistent with oligosaccharide **8**, demonstrated by Aspinall and Nasir-Ud-Din[8], and with structure **9** demonstrated by Aspinall and Sanderson[9].



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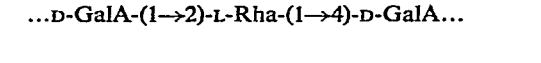

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The carbohydrate-utilization pattern exhibited by *Cephalosporium sp.* provides evidence for chain types in addition to Chain I. After the initial large decline in carbohydrate, a relatively long lag-period occurred, followed by a second substantial decline of carbohydrate at 245–337 h. This would indicate the presence of two (or more) additional chain-types, the one that was used beginning at 245 h and another (or others) that remained at 337 h.

The material left could resemble a limit dextran. The portion degraded in this instance would be cleaved off by "exo"-enzymes of oligomerases until a branch (or linkage) was reached that blocked the attacking enzymes.

This possibility was also suggested by the results of fractionation on Sephadex G-50 of the samples taken at 218, 245, 292, and 337 h (Figs. 3a and 3b). A second peak of material having a molecular weight less than 10,000 began to appear, at the expense of the excluded peak, at 218 h and reached a maximum at 292 h. The fact that no material of lower molecular weight was found means that any cleavage occurring was "endo" in nature. At 337 h, the second peak was again a shoulder on the first peak, which also had decreased slightly. At 292 h, the percentage of uronic acid present in the tip of the excluded peak (fraction 9) was 24%, whereas that in the second peak (fraction 12) was 30%. This low uronic acid content at this point is confirmed by Fig. 5. The values in Fig. 5 are high because they were not corrected to indicate uronic anhydride, but they do indicate the trend in content of uronic acid. Fig. 1 also confirms this low uronic acid content, showing that uronic acid disappeared 24 h before neutral sugar (at *ca.* 269 h).

The evidence presented here is somewhat difficult to interpret. Again no material of low molecular weight appeared in the culture fluid, yet the uronic acid and neutral sugars were used entirely separately. This observation could indicate one of two situations. Either the chain structure of the material utilized was exactly opposite to that proposed for Chain I, namely, a neutral chain with uronic acid branches, or it consisted of two completely separate chains, one of uronic acid and one of neutral sugar. The results of Aspinall and his various coworkers[8–10] would seem to argue against the former possibility. However, further examination of their results suggests that, by arranging the structure **10** (demonstrated by Aspinall and Sanderson[9]) as shown in **11**, it is possible to arrive at a different interpretation.

O-(GalA)-(1→2)-*O*-(L-Rha*p*)-(1→4)-D-Gal **10**

...4)-α-L-Rha-(1→4)-D-Gal... **11**
2
↑
1
α-D-GalA

By adding more α-D-galactosyluronic acid -(1→2)-L-rhamnose residues to the nonreducing end of **11**, a structure such as **12** would be formed.

...4)-α-L-Rha-(1→[4)-α-L-Rha-(1]$_2$→4)-L-Rha-(1→4)-D-Gal... **12**

2	2	2	.
↑	↑	↑	.
1	1	1	.
α-D-GalA	α-D-GalA	α-D-GalA	(α-D-GalA?)

Structure **12** as shown, is not compatible with the results of Aspinall and Nasir-Ud-Din[8], who found no trace of nonreducing D-galacturonic acid residues upon hydrolyzing the methylated, carboxyl-reduced polysaccharide. However segments of structure **7**, Chain I, could be linked to O-4 of the D-galacturonate branches of structure **12**, as shown in structure **13**.

...4)-α-L-Rha-(1→[4)-α-L-Rha-(1]$_2$→4)-α-L-Rha-(1→4)-D-Gal... **13**

2	2	2	.
↑	↑	↑	.
1	1	1	.
α-D-GalA	α-D-GalA	α-D-GalA	(α-D-GalA?)
.	4	.	.
.	↑	.	.
.	1	.	.

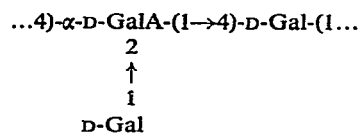
[β-D-Gal-(1→2)-α-D-GalA]$_4$
4
↑
1
...2)-α-L-Rha-(4...

Chain I could be removed by the initial "endo" enzymes and degraded as described previously, leaving structure **12**.

That the neutral portion of this second chain was mostly rhamnose is evident from the g.l.c. data presented in Table I. At 292 h, the ratio of rhamnose to galactose was almost 7:1 by weight. At 529 h, the ratio was 2:1. This indicates that the rhamnose to galactose ratio in Chain II was *ca.* 4:1. This means that Chain II as a whole contained roughly 50% uronic acid (probably as galacturonic acid), 40% rhamnose, and 10% galactose.

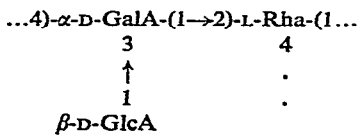
Chain II may have had at least 50 monosaccharide residues, because no fragments of low molecular weight (below 8,000–10,000) were evident by chromatography on Sephadex G-50. Equally probable, however, is the attachment of short lengths of Chain II between larger fragments of the material remaining at 360 h, namely Chain III. "Endo" cleavage occurring between the ends of Chains II and III would leave Chain II exposed for attack. Such "endo" cleavage would most probably take place at the nonreducing end of Chain II, as its rhamnose residues could then be attacked by the "exo"-α-L-rhamnosidase produced at *ca.* 245 h. An "exo"-α-D-galacturonate hydrolase would be necessary to cleave the galactosiduronic acid branches from the rhamnose. Such an enzyme was at no time evident during the growth of *Cephalosporium sp.*, and was not found when the *Cephalosporium* enzymes were separated and purified[11]. An "exo"-lyase was found[11], but such an enzyme would not be able to attack these branches because "exo"-lyases attack from the reducing end of the chain[15].

Not much may be stated definitely concerning Chain III, except that the material present contained uronic acids and neutral sugars in approximately the same ratio as the original polysaccharide. The molecular weight of the material ranged from under 100,000 to slightly below 10,000. Again no material of lower molecular weight was evident. It is possible that this chain may contain internal, α-linked D-galactose residues in a chain such as **14** proposed by Aspinall and Sanderson[10]; the only α-D-galactosidase found was strictly an aryl α-D-galactosidase. However, the induction of an aryl α-D-galactosidase is not necessarily indicative of the presence of an α-D-galactosidic linkage. As no β-D-glucosiduronase activity was found, and as no low molecular-weight material was present that could contain D-glucosiduronic acid, it is highly probable



14

that most of the glucosiduronic acid is contained in Chain III. With slight modification, structure **14** and structure **15** (demonstrated by Aspinall and Nasir-Ud-Din[8]) can be connected to form a repeating unit as shown in **16**. An obvious



15

...[4)-α-D-GalA-(1→2)-L-Rha-(1]$_3$→4)-α-D-GalA-(1→4)-D-Gal...
3 4 2
↑ ↑ ↑ **16**
1 1 1
β-D-GlcA α-L-Rha β-D-Gal

question arises concerning this structure; why is this chain not split by the "endo" enzymes which split the other chains? Chain III proposed here is a complex chain in which no two consecutive units or linkages are alike. Very specific "endo" enzymes would be necessary to degrade such a structure, and "exo" cleavage would require at least four different enzymes to be present simultaneously. As no β-D-glucosiduronase was demonstrably produced by the organism, β-D-glucosiduronic acid branches would effectively block "exo" cleavage from the nonreducing end of the molecule. Hence a structure such as **16** would be very resistant to microbial attack. This structure accords reasonably with the data shown in Table I for the neutral-sugar composition of the sample at 529 h, and it contains *ca.* 50% of uronic acid residues.

The chains postulated here could be arranged in a number of ways. Aspinall and Sanderson[10] considered that the two structures (**1** and **2**) are repeating units in a single chain (**3**). We find no real evidence to support this conclusion. Our evidence indicates the presence of at least three chain types. These chains could be repeating units in a single chain, but they could just as easily be arranged as two parallel chains bridged at intervals by the third chain. We have discussed one possibility already above in connection with structure **13**. Studies with the appropriate enzymes could shed a great deal of light on the gross structural characteristics of the gum.

During the course of this study, a number of factors became evident concerning the resistance of gum karaya to microbial attack. We found[11] that the crude gum contains an inhibitory agent, probably poly-flavan-3-ol or poly-flavan-3,4-diol bound to polysaccharide material. This inhibition could eventually be overcome by most organisms to the extent that they could utilize monosaccharides in the presence of the crude gum; however, none of the organisms tested except the *Cephalosporium sp.* were able to attack even the purified polysaccharide after the inhibitory fraction had been removed.

We consider that our data eliminate the hypothesis that the tertiary structure of the polysaccharide is the sole source of resistance once the inhibitory agent has been removed. *Aspergillus niger* was unable to attack the purified polysaccharide in the *Cephalosporium* culture fluid, even in samples taken at the time that major utilization of carbohydrate by *Cephalosporium sp.* was occurring. Obviously, at this time the original polysaccharide was considerably degraded and areas of Chain I were sufficiently exposed for *Cephalosporium* to utilize them. *Aspergillus niger* is a known source of many glycosidases, including those that are seemingly necessary to cleave the linkages present in the gum[16–25]. The resistance of the gum to *A. niger* is thus apparently based on two facts: (*1*) even if *A. niger* is capable of producing all the required enzymes, the proper inducers must be present to cause the secretion of these enzymes; and (*2*) as shown by a study in which Pectinol 10-M (from *A. niger*) was

shown to attack the gum only in the presence of *Cephalosporium* enzymes[11], not only must some of the proper enzymes be induced, but all of the necessary enzymes must be present at the proper times.

Cephalosporium was able to cause initial fractionation because the proper enzymes were initially produced. *Cephalosporium* was further able to degrade a large portion of the fragment chains resulting from "endo" cleavage, because these fragments induced all of the enzymes necessary for their degradation. The material that was left (Chain III) was probably not attacked because *Cephalosporium* was not capable of producing, among possible other enzymes, a β-D-glucosiduronase.

Synergistic effects were observed between purified *Cephalosporium* enzymes and Pectinol 10-M[11], which is good evidence that, with an additional enzyme or enzymes present, *A. niger* enzymes could degrade the gum. Whether it was merely the lack of one or more enzymes, or whether the wrong enzymes were induced in the case of the actual culture of *A. niger*, is not evident.

It is apparent, however, that *A. niger* was unable to produce the proper complement of enzymes to attack the complex polysaccharide at any stage of degradation by *Cephalosporium*. Thus we consider that resistance of the gum is fully a matter of enzyme-induction properties.

EXPERIMENTAL

Substrates. — Crude gum karaya (60 g), obtained as a gift from Stein Hall and Co., Inc., New York, New York (Control No. 3-1897), was dispersed in 500 ml of 95% ethanol in an 8-liter Pyrex battery-jar. The jar was agitated on a 20-cm stroke reciprocal-shaker and 1500 ml of sodium hydroxide solution was added (final concentration of base, 0.2M) to deacetylate the gum. After shaking for 45 min at room temperature, with occasional scraping down of the jar sides, the mixture was adjusted to pH 2.5 with 6M hydrochloric acid with vigorous stirring. A thick orange gel formed. The jar was covered with aluminum foil and heated for 20 h in a constant-temperature (97°), constant-volume water biancher. The resulting clear yellow solution was filtered while hot to remove the reddish-brown phlobaphen precipitate, and then cooled to room temperature.

To the filtrate in an 8-liter battery jar was added three volumes of 95% ethanol, the mixture was stirred well, and the precipitate was allowed to settle overnight. The pink supernatant solution was siphoned off and discarded. The white gummy layer in the bottom of the jar was formed into a ball and the occluded liquid was expressed by squeezing in a cheese cloth. The ball was broken up into small pieces and placed in 400 ml of abs. ethanol, whereupon the gum became brittle and fibrous. The gum was shredded further in the ethanol and then collected by filtration, washed with abs. ethanol and then acetone, and air dried. The dry gum was ground to a fine powder in a mortar and then redissolved in 100 ml of water and reprecipitated with one volume of 95% ethanol. The precipitate was treated as before, ground, and dried overnight *in vacuo*. A yield of 37 g (61.7%) of the purified gum was obtained.

Aliquots of a solution of the purified gum in water were passed over a column (2.5 × 100 cm) of Sephadex G-100 and the excluded material (molecular weight > 100,000) was collected and precipitated as before. The molecular weight of the excluded fraction was estimated to be approximately 550,000 by the Nelson–Somogyi reducing-group assay[12].

p-Nitrophenyl α-D-galactopyranoside, β-D-glucosiduronic acid, and β-D-galactopyranoside were obtained from Pierce Chemical Company, Rockford, Illinois. *p*-Nitrophenyl α-L-rhamnopyranoside was prepared by the method of Kamiya, Esaki, and Hama[26] from tri-*O*-acetyl-L-rhamnosyl bromide prepared according to Fischer, Bergmann, and Rabe[27].

Melibiitol was prepared from melibiose (1 g) in water (3 ml) by adding sodium borohydride (0.1 g) in 2 ml of water. After 30 min at room temperature, the borohydride was decomposed by acidifying to pH 3.5 with Dowex-50 (H^+). The resulting boric acid was removed as methyl borate by repeated evaporation of methanol under diminished pressure. The product was freeze dried, yielding 670 mg (70%).

Reduced β-D-glucuronan was prepared from oxidized cellulose (Eastman Chemical Products, Inc., Kingsport, Tenn.; Control No. 3477; 18–22% carboxyl) in the same way. D-Galacturonan was obtained from Sunkist Growers, Corona, California (Lot No. 3491).

Analyses. — Total carbohydrate was assayed by the phenol–sulfuric acid method of Dubois *et al.*[13], uronic anhydride by the carbazole method of McComb and McCready[14], and neutral sugars by the anthrone method of Dische[28]. Reducing sugar was determined by the Nelson colorimetric modification of the Somogyi copper method, as described by Hodge and Hofreiter[12].

Gas–liquid chromatography. — A Varian Aerograph 1740 gas chromatograph equipped with dual flame-ionization detectors was used for this study. Monosaccharides and monouronic acids were determined by the method of Raymond and Nagel[29], using 1.5% SE-30 instead of the 0.5% used by Raymond and Nagel. For separation of the monomers as their *O*-trimethylsilyl derivatives, an initial oven-temperature of 150° was used, followed after 2 min by a 4°/min linear temperature-program to 220°. Methyl tri-*O*-(trimethylsilyl)-α-L-rhamnoside was used as an internal standard. All injections (1 μl) were made with a Hamilton CR-700-20 constant-rate, constant-volume syringe.

Studies with Cephalosporium sp. — *Cephalosporium sp.* was an air contaminant initially isolated from crude deacetylated gum karaya by streaking on acidified potato dextrose agar plates; it was then stored on a slant of the same medium.

A basal mineral medium for fungi was prepared according to Lilly and Barnett[30]. Ammonium nitrate (1.74 g/l) was used as the nitrogen source instead of asparagine, as the latter could also serve as a carbon source. Calcium was also added as calcium nitrate (21.5 mg/l). Yeast extract and the mineral salts were sterilized separately from the carbon sources. The organism was kindly classified as to genus by Dr. Jack Rodgers of the Washington State University Plant Pathology Department.

In order to determine the sequence of monosaccharide utilization by *Cephalo-*

sporium sp. and *Aspergillus niger*, 50 ml of basal mineral medium containing 0.5% each of D-galactose, D-glucuronic acid, D-galacturonic acid, and L-rhamnose were added to each of two 250-ml Erleneyer flasks via a Gelman bacteriological filter. The two flasks were inoculated from slants with the respective organisms and zero-time samples of 1.5 ml were taken immediately. The cultures were incubated at 25° and sampled every 12 h.

The samples (1.5 ml) were immediately filtered on 2-ml, fine sintered-glass filters to remove mycelia. One ml of each filtrate was then placed into a 25-ml round-bottom flask and evaporated to dryness *in vacuo*.

For gas chromatography, the samples were dissolved in 1 ml of *N,N*-dimethylformamide containing 2.5 mg/ml of methyl α-L-rhamnoside, silylated, and chromatographed as described already. The injector and detector temperatures were 290°.

The sequences of enzyme production and carbohydrate utilization were determined as follows: A mother culture was prepared by growing *Cephalosporium sp.* for 4 days at 30° on 20 ml of sterile 0.5% D-glucose–basal mineral medium in a 2.8-liter Fernbach flask equipped with a surface aerator and sampling tube, as shown in Fig. 6.

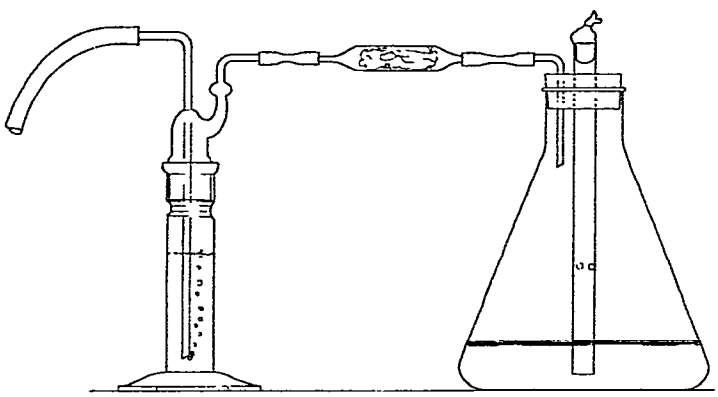


Fig. 6. Apparatus used for the culture of *Cephalosporium sp.* on purified gum Karaya.

After 2.5 weeks, when a thick pellicle had formed, the glucose was drawn off by a sterile siphon tube inserted through the sampling tube, and was replaced by 1 liter of 1% purified gum–basal mineral medium.

Two 10-ml samples were immediately taken aseptically, and each was filtered through a fine, sintered-glass filter into a screw-cap culture tube. One sample was immediately frozen and the second refrigerated with a drop of toluene added as a preservative. Two samples were aseptically removed every 12 h at first and then at longer intervals, and treated as before.

Pectic-enzyme activity was determined by measuring the reduction of viscosity of pectic acid. Viscosity was measured with an Ostwald viscometer (water flow, 59.5 sec) immersed in a water bath at 25°. The reaction mixture contained 1% pectic

acid, 0.1M acetate buffer (pH 5.0), and 1 ml of enzyme in a total volume of 3 ml. When the viscosity had reached one-half of its original maximum, the sample was boiled 2 min and the reducing power determined on a 1-ml aliquot by the Nelson–Somogyi assay[12].

Melibiitol, lactitol, and reduced β-D-glucuronan (2mM each and 0.2M in acetate buffer, pH 5.0) were incubated with an equal volume of enzyme, and 1-ml samples were assayed by the Nelson–Somogyi assay.

For *p*-nitrophenyl glycoside assays, the assay mixture contained mM *p*-nitrophenyl glycoside, 0.1M phosphate buffer (pH 5.0), and 1 ml of enzyme in a total volume of 3 ml. The assay mixtures were incubated in stoppered tubes in a 25° bath. Just prior to measuring the $O.D._{410nm}$, 0.1 ml of M sodium hydroxide was added to each assay mixture. Substrate and enzyme blanks were also determined.

In the case of pectic-enzyme activity, the activity is expressed as μmoles of reducing power (D-glucose equivalent) released per h per ml of enzyme. In the case of the *p*-nitrophenyl derivatives, activity is expressed as μmoles of *p*-nitrophenol ($\varepsilon = 14{,}370$) released per h per ml of enzyme.

Carbohydrate assays were performed on 1:100 dilutions of each sample. One ml of diluted sample was used for the carbazole and phenol–sulfuric acid assays, and 2 ml was used for the anthrone assay.

In order to determine the ratio of rhamnose to galactose in the neutral fraction utilized by *Cephalosporium sp.*, samples of deacetylated, crude gum, and the culture fluid (130, 292, and 529 h) were hydrolyzed by the method of Albersheim *et al.*[31]. The residue of each sample, after hydrolysis and evaporation, was dissolved in 1 ml of *N*,*N*-dimethylformamide containing 2 mg/ml of methyl α-L-rhamnoside. The *O*-trimethylsilyl derivatives were prepared, and assayed by gas–liquid chromatography as already described[29].

Attempt to grow Aspergillus niger *on* Cephalosporium-*degraded polysaccharide.* — *Aspergillus niger* spores from a potato dextrose agar slant were suspended in 10 ml of sterile water. One-ml aliquots of the 12-, 36-, 96-, 195-, and 360-h samples from the *Cephalosporium* degradation of the purified polysaccharide were sterilized by autoclaving in screw-cap culture tubes, cooled, and inoculated with 0.1 ml of the spore suspension. Zero-time samples were prepared in the same way by using a boiled-spore suspension. After one week of incubation at 30°, 0.1 ml of each sample was diluted to 1 ml and the carbohydrate was determined by the phenol–sulfuric acid assay[13].

Sephadex chromatography of Cephalosporium-*degraded fraction D.* — The G-100 column used in this study was 2.5 × 100 cm, having a bed volume of 480 ml and a void volume of 156 ml, as determined by passing 1 ml of 1% Pharmacia Blue Dextran 2000 (Pharmacia Fine Chemicals, Piscataway, N.J.) having a molecular weight of 2 million over the column. The G-50 column was 2.5 × 45 cm, having a bed volume of 200 ml and a void volume of 60 ml, again determined by using Blue Dextran. Water was used as the eluant with both columns.

In order to fractionate the polysaccharide, 2 ml of each sample to be determined was carefully added to the top of the gel bed and drained into the bed. Elution with

water was begun, and 6.3-ml fractions were collected. Samples from each fraction (0.01 ml) were analyzed by the phenol–sulfuric acid assay ($O.D._{480nm}$) for total carbohydrate, and by the carbazole assay (0.02 ml) for uronic acid ($O.D._{520nm}$).

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REFERENCES

1 G. O. Aspinall, *Pure Appl. Chem.*, 14 (1967) 43.
2 F. Smith and R. Montgomery, *The Chemistry of Plant Gums and Mucilages and Some Related Polysaccharides, Amer. Chem. Soc. Monograph Ser.*, Reinhold Pub. Corp., N.Y., 1959.
3 L. Beauquesne, *Compt. Rend.*, 222 (1946) 1056.
4 G. O. Aspinall and J. Baillie, *J. Chem. Soc.*, (1963) 1702.
5 C. W. Nagel, Unpublished results.
6 J. V. Kubal, *Coll. Czech. Chem. Commun.*, 13 (1948) 378.
7 J. V. Kubal and N. Gralen, *J. Colloid Sci.*, 3 (1948) 457.
8 G. O. Aspinall and Nasir-Ud-Din, *J. Chem. Soc.*, (1965) 2710.
9 G. O. Aspinall and G. R. Sanderson, *J. Chem. Soc.*, (*C*), 16 (1970) 2256.
10 Ref. 9, p. 2259
11 W. R. Raymond, Ph. D. Thesis, Washington State Univ., Pullman, Wash., 1971.
12 J. E. Hodge and B. F. Hofreiter, *Methods Carbohyd. Chem.*, 1 (1962) 383.
13 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350.
14 E. A. McComb and R. M. McCready, *Anal. Chem.*, 24 (1952) 1630.
15 J. D. MacMillan, H. J. Phaff, and R. H. Vaughn, *Biochemistry*, 3 (1964) 572.
16 Y. C. Lee and V. Wacek, *Arch. Biochem. Biophys.*, 138 (1970) 264.
17 O. P. Bahl and K. M. J. Agrawal, *J. Biol. Chem.*, 244 (1969) 2970.
18 S. Kamaya, S. Esaki, and M. Hama, *Agr. Biol. Chem.*, 31 (1967) 133.
19 P. J. Mill, *Biochem. J.*, 99 (1966) 557.
20 Ref. 19, p. 562.
21 P. J. Mill and R. Tuttobello, *Biochem. J.*, 79 (1961) 57.
22 R. Tuttobello and P. J. Mill, *Biochem. J.*, 79 (1961) 51.
23 A. Koller and H. Neukom, *Eur. J. Biochem.*, 7 (1969) 485.
24 P. Albersheim, H. Neukom, and H. Deuel, *Helv. Chim. Acta*, 43 (1960) 1422.
25 P. Albersheim and U. Killias, *Arch. Biochem. Biophys.*, 97 (1962) 107.
26 S. Kamiya, S. Esaki, and M. Hama, *Agr. Biol. Chem.*, 31 (1967) 397.
27 E. Fischer, M. Bergmann, and A. Rabe, *Ber.*, 53 (1920) 2362.
28 Z. Dische, *Methods Carbohyd. Chem.*, 1 (1962) 488.
29 W. R. Raymond and C. W. Nagel, *Anal. Chem.*, 41 (1969) 1700.
30 V. G. Lilly and H. L. Barnett, *Physiology of the Fungi*, McGraw–Hill, New York, 1951.
31 P. Albersheim, D. J. Nevins, P. D. English, and A. Karr, *Carbohyd. Res.*, 5 (1967) 340.