CHARACTERIZATION STUDIES ON AN EXTRACELLULAR HETEROPOLYSACCHARIDE PRODUCED BY *ANABAENA FLOS-AQUAE* A-37

by

EDITH BOYANTON DAVIS

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy
in the Department of Microbiology

State College, Mississippi

January, 1970

NGR-25-001-004

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ACKNOWLEDGEMENTS

The writer would like to express her appreciation to Dr. R. G. Tischer, Professor and Head of the Department of Microbiology for the opportunity to conduct these investigations, for his patience and consideration and for the guidance and constructive criticisms of these research efforts. Appreciation is also directed to Dr. Lewis R. Brown for his many helpful criticisms and suggestions and to Dr. John C. Mickelson of the Department of Microbiology.

The writer would like to express her gratitude to the readers of this dissertation for their many helpful suggestions and criticisms.

She would also like to express her appreciation to her husband without whose patience, encouragement and understanding this work could not have been undertaken and completed.

The writer is also indebted to the National Aeronautics and Space Administration, National Science Foundation and Mississippi State University for providing financial assistance during these investigations.

E. B. D.

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INTRODUCTION

During recent years much attention and research has been directed toward polysaccharides produced by the marine algae. This is quite understandable since many of these polysaccharides are of economic importance. One of these polysaccharides, agar, is of great importance since it is utilized in both medical and microbiological research, and in industry. Likewise, the polysaccharides in the forms of mucilages and gums which are produced by many higher plants have received considerable attention because they are of economic importance.

On the other hand, the polysaccharides produced by the fresh-water algae have not received an adequate amount of attention; therefore, only a minute amount of information is available concerning them either generally or specifically. Horton and Wolfrom (1963) state that heteropolysaccharides which contain both neutral sugars and uronic acids comprise one of the most complex areas of carbohydrate chemistry and that the information pertaining to them is most incomplete. Most of the existing information was obtained from gums and mucilages of higher plants. The quantity of knowledge pertinent to heteropolysaccharides of microorganisms, especially those of the fresh-water algae, is at the present insufficient.

These studies were undertaken because a fresh-water alga which was known to be an excellent polysaccharide producer was available. This organism had been the subject of numerous previous publications; therefore, its characteristics and peculiarities were well known. The investigations to be reported in this dissertation were undertaken to attempt to elucidate the characteristics of an extracellular heteropolysaccharide produced by a bacteria-free culture of the blue-green alga, Anabaena flos-aquae A-37. These studies were concentrated on the intact polymer and relatively little work was done on the constituents.

LITERATURE REVIEW

Over the past half century polysaccharides produced by biological systems have attracted the attention of many investigators. Most of these early inquiries into the polysaccharides were the result of curiosity, although from this approach have come the much more serious endeavors.

During the search of the literature, several applications for plant and algal polysaccharides were encountered which might be worth mentioning. In Russia, Koz'mina and Baronova (1968) have investigated the use of colloidal polysaccharides of marine algae in the baking industry. They found that when carrageenan was added to dough prepared from soft wheat the gluten properties improved during both the processing and the baking. Agaroid and Na alginate were found to have a lesser effect, whereas agar had no effect.

Gudjons (1969) has surveyed many water-soluble polysaccharides and has listed 24 economically important natural plant polysaccharides according to their properties and economic field of use.

Several Japanese workers have found that a polysaccharide extracted from Chlorella and Scenedesmus has the ability to stimulate the reticuloendothelial system in

rats (Yamada, et al., 1969; Tohoku, 1969).

A variety of books and periodical articles on polysaccharides is available; however, upon investigation one finds that the vast majority of this information is pertinent only to the gums and mucilages produced by higher plants (Jones and Smith, 1949; Whistler and Smart, 1953; Smith and Montgomery, 1959). Smith and Montgomery (1959) list several algal heteropolysaccharides which have been analyzed and characterized in terms of constituent monosaccharides, but none of these has been characterized to such an extent as to make it a definable natural product. The majority listed are polysaccharides produced by marine algae.

Algal polysaccharides were completely ignored by Stacey and Barker (1960) in their consideration of microbial polysaccharides. They discussed polysaccharides produced by the bacteria, the yeasts and yeast-like fungi, the molds and the protozoa. Evidently they did not consider the algae to be microorganisms.

Microbial polysaccharides were reviewed by Jeanes (1968). Algal mucilages and extracellular products have been briefly considered by Lewin (1962). Percival and McDowell (1967) have prepared a very excellent account of the marine algal polysaccharides. It remains for the counterpart on fresh-water algal polysaccharides to be

compiled.

There are available numerous techniques for undertaking the study of polysaccharides (Whistler and Smart, 1953; Smith and Montgomery, 1959; Percival and McDowell, 1967). The application of most of these procedures to the study of fresh-water algal polysaccharides is limited because most of the methods are applicable at the present to relatively simple polymers composed of usually one monosaccharide. Overton (1963) has compiled a good review dealing with the isolation and purification of natural products including polysaccharides. Procedures for the determination of physical properties of solutions of polysaccharides have been outlined by Banks and Greenwood (1963). Bouveng and Lindberg (1960) have outlined the techniques for the determination of the structure of polysaccharides. Extreme caution would have to be exercised in attempting to apply some of these methods to the study of algal heteropolysaccharides, particularly in the interpretation of the data.

Whistler and Wolfrom (1962, 1963) have compiled many of the methods utilized in the study of carbohydrates. Their usefulness is primarily in the field of monosaccharides with some emphasis on polysaccharides.

Jones (1953) was apparently the first to employ a detergent in the isolation of a biological product. He

utilized Cetyltrimethylammonium bromide (Cetavlon[1]) for the isolation of bacterial nucleic acids. He further showed that Cetavlon could also be applied in the separation and purification of bacterial polysaccharides.

Scott (1955) later observed that Cetavlon could be used to precipitate acidic non-sulphated polysaccharides. In 1957, Barker, Stacey and Zweifel, demonstrated that Cetavlon could be utilized to precipitate borate complexes of neutral polysaccharides. They were able to separate a mixture of yeast mannan and glycogen with a recovery of 82 and 80 percent, respectively. Glycogen and inulin were separated with an efficiency of 69 and 58 percent, respectively.

Cetavlon was employed in structural studies of the capsular polysaccharide and an acidic polysaccharide produced by Aerobacter aerogenes (Barker, et al., 1958a, 1958b). They further found that Cetavlon could be employed to separate nucleic acids from polysaccharides. Both substances are precipitated at a neutral pH with Cetavlon; however, the precipitated acidic polysaccharides are soluble in 0.25 M NaCl, whereas the nucleic acid complex is insoluble.

Jones and Painter (1959) acetylated and subsequently

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[1]The specific trade name for the Cetyltrimethylammonium bromide that he utilized.

fractionated the hemicellulose from loblolly pine and were able to demonstrate two distinct fractions. The first chloroform soluble fraction of the hemicellulose acetate was found upon deacetylation and hydrolysis to contain galactose, glucose and mannose. The second chloroform soluble fraction was found to contain xylose, 4-O-Methyl-glucuronic acid and 2-O-(4-O-Methyl glucuronosyl) xylose.

The structure of the heteropolysaccharide gum produced by Acacia nubica was found to consist of a highly branched framework of D-galactose to which were attached D-glucuronic acid residues and side chains containing L-arabinose (Anderson and Cree, 1968).

A large amount of work has been done on the polysaccharides produced by bacteria. Numerous reports are available concerning bacterial polysaccharides with respect to their antigenic properties. Davies (1955) has studied the polysaccharides produced by some Gram negative bacteria including the genera Salmonella and Shigella. He also studied a specific polysaccharide produced by Pasteurella pestis and found it to be a lipopolysaccharide containing glucose, glucosamine and an unidentified aldoheptose.

Wang, Steers and Norris (1963) found that Lactobacillus bifidus produced an extracellular polysaccharide composed of galacturonic acid, galactose, glucose and

6-deoxytalose. No further studies were undertaken to elucidate the characteristics of the polysaccharide.

The extracellular polysaccharide produced by Serratia marcescens was found by Young and Adams (1965) to be, in reality, two polysaccharides. They were able to effect a separation utilizing Cetavlon. The Cetavlon-precipitated polysaccharide contained glucose, mannose and uronic acid. The polysaccharide retained in the supernatant was composed of glucose, rhamnose, a heptose, hexosamine, uronic acid and traces of both fucose and mannose.

The first fresh-water algal polysaccharides were apparently isolated by Payen (1938). He extracted the extracellular polysaccharides from three members of the family Nostocaceae: Rivularia bullata, Calotrix pulvinata and Nostoc commune. However, he did no further studies on these polysaccharides.

In 1943, Kylin extracted a polysaccharide from the cells of Calotrix scopulorum which was found to contain galactose, a pentose and sulfuric acid.

Probably the first extensive work on fresh-water algal polysaccharides was done by Fredrick (1951) when he isolated a polysaccharide from Oscillatoria princeps which was determined to be a glycogen-like polymer. In 1952, he discovered a polysaccharide variant of O. princeps

which produced a polysaccharide similar to plant amylose in that it was an almost completely linear polymer of glucose. He suggested that this variant was the result of a mutation which effected a decrease in the quantity of the branching enzyme essential for the formation of the glycogen-like polymer produced by the parent type. Later he found that polysaccharide variants of this type could be induced by subjecting the alga to low temperatures (5°C) for about 30 days; however, these variants would revert to normal upon return to 25°C (Fredrick, 1953a). Fredrick (1953b) also investigated the branching characteristics of the polysaccharide produced by O. princeps and the kinetics of polysaccharide formation (Fredrick, 1954).

Hough, Jones and Wadman (1952) undertook the investigation of the polysaccharides produced by some fresh-water algae. Nitella was harvested from Lake Windermere and definitely was not a uniform sample; however, the main carbohydrate component was found to be a cellulose. Oscillatoria and Nostoc were collected at the Freshwater Biological Association, Ambleside, England. The conditions under which these algae were cultivated were not described, but it may be assumed that they were not axenic cultures. The polysaccharide produced by Oscillatoria was found to be a polyglucosan of the amy-

lopectin type. The polysaccharide produced by Nostoc was completely different from the others in that it was found to contain five neutral sugars: rhamnose, xylose, glucose, galactose and an unknown sugar; and both glucuronic and galacturonic acids. The validity of these observations is open to question because of the impurity of the samples.

Bishop, Adams and Hughes (1954) investigated the polysaccharide produced by Anabaena cylindrica. The purity of the culture with respect to bacterial contaminants was not stated. The polysaccharide was found to be a complex heteropolysaccharide composed of glucose, xylose, galactose, rhamnose, arabinose and glucuronic acid in a molar ratio of 5:4:1:1:1:4. The polysaccharide was also demonstrated to be chemically homogeneous.

Studies on the extracellular polysaccharides produced by 15 species of the genus Chlamydomonas were conducted by Lewin (1956). The more frequently encountered monosaccharides were galactose and arabinose with glucose, xylose, fucose, mannose and uronic acids occurring to a lesser extent.

A polysaccharide produced by Nostoc muscorum was found to contain galactose, glucose, xylose, arabinose, ribose, rhamnose and two unidentified sugars (Biswas, 1957). Studies were also conducted to elucidate the

metabolic pattern of polysaccharide synthesis utilizing C^{14}-labeled substrates in the presence of light.

Eight species of green and blue-green algae in pure culture were investigated by Moore and Tischer (1964) in order to determine the quantity of polysaccharide produced as well as to identify tentatively the components of these polysaccharides. These algae included Chlorella vulgaris, Palmella mucosa, Chlamydomonas sp., Nostoc sp. and Anabaena flos-aquae A-37. In each case the extracellular polysaccharide was found to contain, at least, three monosaccharides. Later Moore and Tischer (1965) studied extensively the extracellular polysaccharide produced by A. flos-aquae A-37. This polysaccharide was reported by them to contain glucuronic acid, glucose, xylose and ribose in a molar ratio of 1:88:39:3. In addition, they investigated the pathway for the biosynthesis of the extracellular polysaccharide.

Maksimova and Pimenova (1966) studied the products excreted by some green algae and found that Chlorella pyrenoidosa and C. vulgaris released polysaccharides composed of galactose, mannose, arabinose, xylose, ribose, fucose and rhamnose. Traces of sucrose, glucose and fructose were found as free sugars in the spent medium. These polysaccharides were released especially during the stationary phase of growth.

Osetrov, Shnyukova and Vlasishing (1969) found that the polysaccharide material produced by Microcystis aeruginosa was very complex and that it varied under natural conditions. It was found to contain acidic polysaccharides, some of which were bound to proteins, and neutral polysaccharides. They did not expand their work to determine the constituents of the polysaccharides.

The polysaccharides produced by 12 strains of blue-green algae were studied by Rashba, Kosenko and Solonin (1969) and were found to contain 11-14 monosaccharides. Anabaena variabilis was demonstrated to have the ability to utilize 359 different carbohydrates. The total carbohydrate content and growth were inversely proportional.

MATERIALS AND METHODS

Culture Medium

Basal Medium

The basal medium employed was a modification of the mineral salts medium described by Dyer and Gafford (1963). The modification consisted of omitting all nitrogen to provide a nitrogen-free medium. There were trace amounts of nitrogen present as a result of the contaminants in the reagent grade chemicals utilized.

Stock solutions	Stock solution concentration grams/liter	Basal medium final concentration grams/liter
A		
K_2HPO_4	25.00	0.2500
H_3BO_3	3.40	0.0340
B		
Ferric citrate	3.00	0.0300
Citric acid	3.00	0.0300
C		
$MnCl_2$	0.50	0.0050
$MgSO_4$	2.50	0.0250
D		
Na_2SiO_3	2.50	0.0250
$ZnSO_4$	0.05	0.0005
E		
$CaCl_2$	6.00	0.0600

The basal medium was prepared by adding 10 ml. of each stock solution to 500 ml. of distilled water and diluting to a liter with distilled water. The pH was adjusted to

8.0 - 8.5 with 10 percent KOH. Microelements were provided by adding 1 ml. of the microelement solution described later. Sterilization was effected at 15 p.s.i. at 121° C for 15 minutes (20 minutes for the large containers).

Nitrogen Sources

The usual nitrogen source employed was KNO_3 at a concentration of 1 gram/liter or 0.138 gram/liter nitrogen. The other nitrogen sources utilized in certain experiments are listed below.

Nitrogen source	Grams/liter	Grams/liter N
NH_4Cl	0.53	0.138
$Mg(NO_3)_2$	1.27	0.138
NH_4NO_3	0.39	0.138
$NaNO_3$	0.84	0.138
$NH_4H_2PO_4$	1.13	0.138
$Ca(NO_3)_2$	0.81	0.138
N_2	----	-----

Microelements

The microelements employed were a modification of those recommended by Gaffron as utilized by Hughes, Gorham and Zehnder (1958). The modification consisted of substituting nitrogen-free compounds for those which contained nitrogen. The molybdenum content was increased from 0.9 p.p.m. to 10 p.p.m.

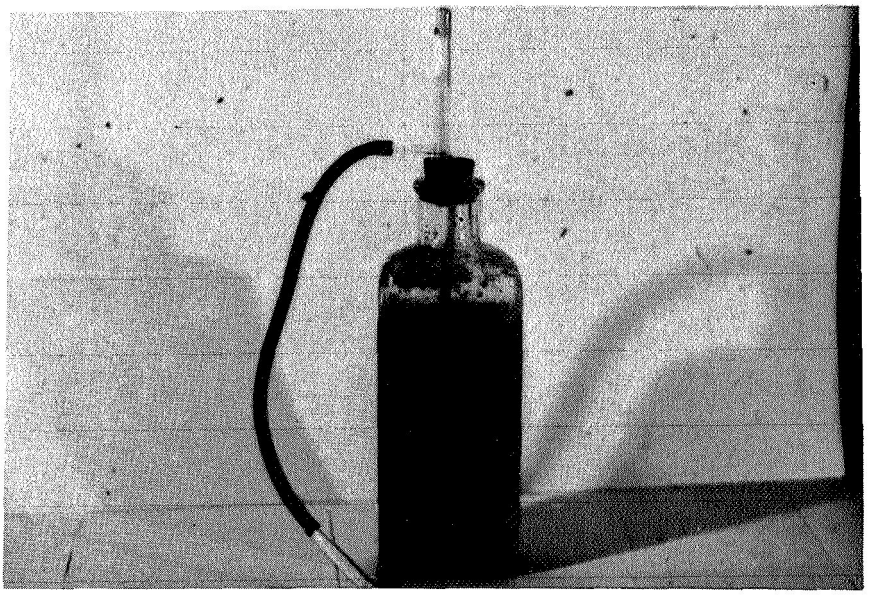
Microelement Stock Solution

Microelement Compound	Milligrams/liter
$MnSO_4 \cdot 4 H_2O$	2.230
$ZnSO_4$	0.287
$Na_2MoO_4 \cdot 2 H_2O$	25.000
$CoCl_2$	0.146
KBr	0.119
KI	0.083
$CdSO_4$	0.154
$NiCl_2 \cdot 6 H_2O$	0.198
V_2O_4	0.020
$Al_2(SO_4)_3K_2SO_4 \cdot 24 H_2O$	0.474
Na_2WO_4	0.033

Culture Chambers

Cultivation of the test organism for inoculum purposes was carried out in 200 ml. prescription bottles equipped with aerators for the supply of 5 percent carbon dioxide. Large scale cultures were grown in either 2 liter Erlenmeyer flasks or 1 liter rectangular bottles equipped with aerators (Figure 1). All culture chambers were fitted with sterile filters for the sterilization of the air entering the cultures. Illumination was provided by two forty watt power groove fluorescent lamps. The intensity of the illumination from the bank of the lamps was 1250 foot-candles.

Figure 1. Apparatus for the Large Scale Cultivation of *A. flos-aquae* A-37.



Culture

A blue-green alga was isolated from a mixed stock culture designated A-37 obtained from a local oxidation pond. The alga was obtained in unialgal culture and purified by Dr. Robert G. Tischer[2] (1965) of all the bacterial contaminants which could be detected by usual laboratory procedures. The alga was subsequently identified as Anabaena flos-aquae by Dr. G. W. Johnston[3]. The alga was designated thereafter as Anabaena flos-aquae A-37[4] to distinguish it from other members of the same species.

The optimum temperature range for the cultivation under laboratory conditions was found to be 37-42° C. Pigment production is suppressed and growth occurs at a minimum below 37° C. Above 42° C bleaching and cell degeneration occur; therefore, all studies were conducted in an incubator regulated at 40 ± 2° C. The duration of the growth period was determined by the purpose of the individual experiment and ranged from 3-4 days for inoculum cultivation for as long as two weeks for polysaccharide production.

Assays for Contaminants

The stock cultures of the alga were routinely examined

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[2]Head, Department of Microbiology, Mississippi State University.

[3]Head, Department of Botany, Mississippi State University.

[4]This alga is number 1444 in the Starr Algal Collection at Indiana University, Bloomington, Indiana.

for the presence of bacterial contaminants. The inoculum was checked 48 hours before use and the culture was checked at the termination of the growth period. All contaminated cultures were discarded. The procedure employed for assaying the cultures was that suggested by Tischer (1965) using nutrient broth. Duplicate nutrient broth tubes were incubated at room temperature and 40°C for the desired length of time. In some cases nutrient agar was employed for the assay. The criterion for determining the purity of the cultures was the absence of bacterial and mold growth in the test medium. Microscopic examinations were also conducted on the cultures.

Purification of Contaminated Cultures

The following procedure was employed whenever it was desired to purify a working culture which had become accidentally contaminated. The purification medium consisted of the basal medium with 0.01 percent KNO_3 and 1.5 percent agar which was poured into sterile petri plates. Dilutions of the alga, prepared with sterile distilled water, were applied to the surface of the solidified medium in one of two ways: (1) streaked with a bacteriological loop or (2) spread over the surface with a curved glass rod. The plates were inverted and incubated at 40°C. Every 24 hours, the plates were examined for algal growth. When growth had occurred, isolated colonies or filaments were picked using

a low power, wide field microscope and sterile Pasteur pipettes. The isolates were placed in test tubes containing 10 ml. of the basal medium and incubated. After growth occurred, the tubes were checked for purity as previously described. In this case the tubes of nutrient broth were incubated for a week before determining the purity of the isolate.

Dry Weight Determinations

Cellular dry weights were obtained by filtering 10 or 15 ml. of a uniform cell suspension which was obtained by blending the culture in a Waring Blendor for 30 seconds onto an asbestos mat in tared Gooch crucibles. The crucibles were dried at 105°C at atmospheric pressure for 24 hours. All weights were obtained using a four place balance.

Cell Count Determinations

The population of some algal cultures was estimated with cell counts. These counts were obtained with a Howard mold counting chamber by counting a sufficient number of fields to provide a representative estimate of cells. Cells/ml. were obtained by multiplying the average number of cells/field by a microscope factor calculated for 450 X magnification (Mickelson, Davis and Tischer, 1967).

Polysaccharide Procedures

Extraction

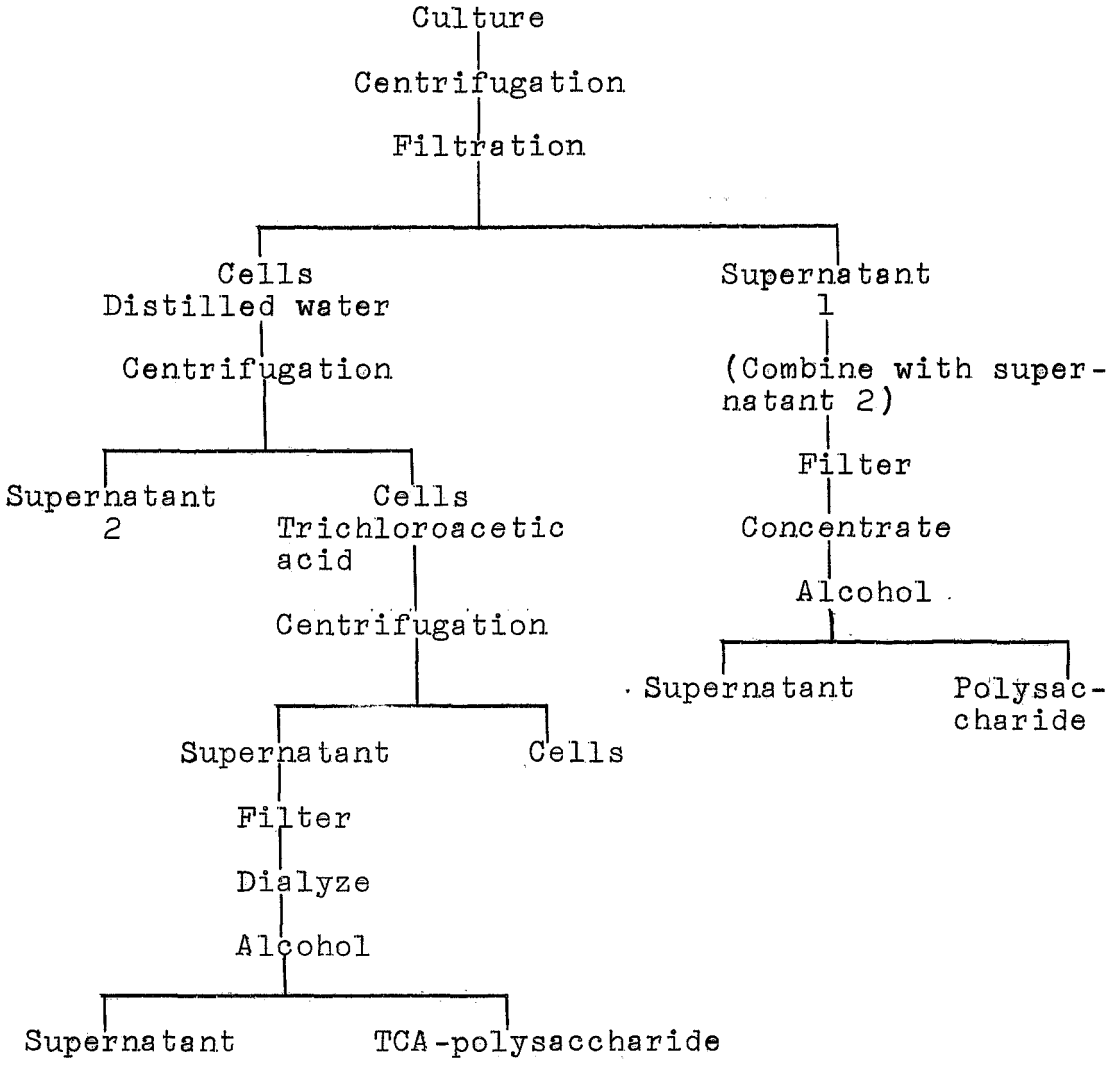
The extracellular polysaccharide was obtained from the spent medium at the termination of the growth period which was usually 10-14 days (Figure 2). The cells were removed from the medium by filtration and centrifugation and then suspended in distilled water for the extraction of adherent polysaccharide. After centrifugation of the cells, the supernatant was combined with the spent medium(2) and filtered. The filtrate was concentrated to one-tenth the original volume in a Buchner flash rotary evaporator at 60°C. The polysaccharide was precipitated with the addition of 2 volumes of absolute ethyl alcohol (Lewin, 1956; Moore and Tischer, 1965).

The washed cells were suspended in distilled water and 1 volume of 10 percent trichloroacetic acid was added. The mixture was refrigerated overnight. The cells were removed by centrifugation and discarded. The supernatant was filtered and dialyzed against distilled water in the cold for 24 hours. The polysaccharide was precipitated with alcohol as previously described. This polysaccharide is referred to as the TCA-polysaccharide.

Purification

The purification procedure consisted of dissolving the precipitated polysaccharide in distilled water and precipitating with 2 volumes of alcohol and repeating the process

Figure 2. Scheme for the Extraction of Polysaccharides from A. flos-aquae A-37.



several times.

After purification, the polysaccharide was dried *in vacuo* at room temperature and ground to a fine powder.

Quantitative Determinations

Extracellular polysaccharide production was quantitated using the phenol-sulfuric acid method as described by Montgomery (1961). The sample was either the spent medium or the precipitated polysaccharide from 10 ml. of the spent medium dissolved in 5 ml. of distilled water. The quantity of polysaccharide was expressed in terms of glucose equivalents.

Quantitative determination of free reducing sugars and monomers in the hydrolyzates was accomplished with Somogyi's arsenomolybdate reagent as described by Nelson (1944).

Standard concentration curves were prepared utilizing reagent grade α-D-glucose.

Hydrolytic Procedures

Cleavage of the polysaccharide was effected with 1 N H_2SO_4 at 100°C for 6 hours in sealed tubes using 4 ml. of the acid for each 50 mg. of the dried purified extracellular polysaccharide. The hydrolyzates were neutralized with solid $BaCO_3$ to the point where the congo red indicator changed color. The $BaSO_4$ was removed by centrifugation and the supernatant was filtered. The hydrolyzates were

then concentrated to about 0.5 ml. *in vacuo* at below 40°C.

Chromatographic Techniques (Kowkabany, 1954)

The components of the polysaccharide hydrolyzate were resolved using either one or two dimensional paper chromatography. Sugar standards were prepared at a concentration of 0.5 percent in 10 percent isopropyl alcohol. For one dimensional chromatography the standards were spotted on the same sheet with the hydrolyzates. Development for both types of chromatography was conducted as descending chromatography in chromatographic cabinets at room temperature. After development and drying at room temperature in a fume hood equipped with a blower, the chromatograms were sprayed with a detection reagent.

Paper

The chromatographic analyses were conducted on 46 x 57 centimeter sheets of Whatman number one chromatographic paper.

Solvent Systems

Solvent A: Phenol-Water-Saturated (Block, Durrum and Zweig, 1958)

The solvent was prepared by mixing 114 ml. of 88 percent liquid phenol with 25 ml. of tap water. The solvent was prepared just prior to use.

Solvent B: Butanol-Acetic Acid-Water (Hough, Jones and Wadman, 1950)

1-Butanol	two volumes
Glacial acetic acid	one volume
Distilled water	one volume

Solvent C: Butanol-Pyridine-Water (Bobbitt, 1963)

1-Butanol	ten volumes
Pyridine	three volumes
Distilled water	three volumes

Detection Reagents

Reagent A: Aniline Hydrogen Phthalate (Partridge, 1949)

Aniline	0.93 gram
Phthalic acid	1.66 grams
Water-saturated 1-butanol	100.00 milliliters

The chromatograms were sprayed and heated at 105°C for about 5 minutes. Pentoses gave a bright red or pink reaction, whereas hexoses were indicated by brown spots.

Reagent B: p-Anisidine-Phthalic Acid (Bobbitt, 1963)

p-Anisidine	1.23 grams (0.1 M)
Phthalic acid	1.66 grams (0.1 M)
Ethyl alcohol (96 percent)	100.00 milliliters

The chromatograms were sprayed and heated at 105°C for 5 minutes. The monosaccharides could be differentiated according to the color reaction as follows: pentoses-- red or pink; hexoses and methylpentoses-- yellow to brown and uronic acids-- pink to brown.

Reagent C: Ninhydrin Reagent (Block, et al., 1958)

Ninhydrin	0.3 gram
Ethyl alcohol (95 percent)	100.0 milliliters

The chromatograms were sprayed and heated at 105°C for 10 minutes. Amino sugars gave a purple color reaction.

Thin-Layer Chromatographic Techniques (Bobbitt, 1963; Stahl,

1964; Randerath, 1963).

Glass plates eight inches square were spread with silica gel G prepared as a slurry in 0.1 N boric acid. The thickness of the layer was about 0.2 mm. The plates were allowed to dry at room temperature after which they were activated by heating at 110°C for 30 minutes. The plates were transferred to a desiccator for cooling and storage.

The solvents and detection reagents employed were the same as those described for paper chromatography.

Identification of Sugars Detected by Chromatographic Techniques

The identity of the sugar spots obtained from the chromatographic analyses of the hydrolyzates was determined by comparison with standard reference sugars. The position of the standard and unknown sugars on the chromatogram was determined by measuring the distance travelel from the origin. This position was expressed as either the R_f value or the $R_{glucose}$ value. The R_f value is the ratio of the distance moved by the sugar to the distance traveled by the solvent front multiplied by 100. The $R_{glucose}$ value is the ratio of the distance traveled by the sugar to the distance traveled by glucose.

Further confirmation of the identity was obtained by preparing duplicate chromatograms and spraying one to serve as a pattern to locate the compounds on the other sheet.

These were cut out and eluted with distilled water. The tentatively identified compounds were then chromatographed alongside of the suspected sugar. Agreement in both color reaction and position was taken as an indication that the compounds were the same.

Fractionation Techniques Employed for the Determination of Homogeneity

Method One: (Smith and Mohtgomery, 1959)

Absolute ethyl alcohol was added to an aqueous solution of the extracellular polysaccharide and the resulting precipitate was removed by centrifugation. This procedure was repeated until no further precipitate was obtained. Each precipitate was then prepared as previously described.

Method Two: (Erskine and Jones, 1956)

The polysaccharide was precipitated from the concentrated spent medium by pouring into two volumes of absolute ethyl alcohol containing 10 ml. concentrated HCl/liter and sufficient ice to keep the reaction at or below room temperature. The precipitate was washed with alcohol to remove the acid and then it was dissolved in distilled water. Any insoluble material was removed by centrifugation. An aqueous solution of cupric acetate (7 percent w/v) was added to the aqueous solution of the polysaccharide, dropwise, until precipitation occurred. This precipitate was removed by centrifugation. Additional cupric acetate solution was added until a maximum concentration of 0.4 percent was

reached. If no precipitate formed, then absolute ethyl alcohol was added portionwise and each precipitate was removed by centrifugation. This was continued until no further precipitate formed. Each precipitate was treated to decompose the copper complex by twice macerating in a Waring Blendor with ethyl alcohol containing 5 percent concentrated HCl (v/v) for about 1 minute. The precipitates were washed with ethyl alcohol until the washings gave a negative test for chloride as detected with a saturated aqueous solution of $AgNO_3$. The precipitates were then dried as described previously.

Method Three:

A. Fractionation of Neutral Polysaccharides (Barker, et al., 1957)

Equal volumes of 10 percent Cetyltrimethylammonium bromide (Cetavlon) (Scott, 1955) and 1 percent borate buffer, pH 8.5 were added to an aqueous solution of the polysaccharide (Zittle, 1951). The resulting precipitate was washed with water, dissolved in 2 N acetic acid and poured into ethyl alcohol. The precipitate was washed with ethyl alcohol and then with petroleum ether and dried.

The same procedure was employed using 1 percent borate buffer, pH 10.

B. Removal of Acidic Polysaccharides (Young and Adams, 1965)

To an aqueous solution of the polysaccharide was added

1/3 volume of 1 percent Cetavlon and sufficient 0.5 N NaOH to make the solution basic. The precipitate was collected, washed and placed in 10 percent acetic acid. Saturated boric acid and Cetavlon were added to the supernatant and any precipitate that formed was removed. The supernatant was then acidified to pH 4.5 with glacial acetic acid, dialyzed against distilled water in the cold for 24 hours and then poured into 5 volumes of absolute ethyl alcohol.

The precipitate which had been dissolved in acetic acid was recovered after dialysis by the procedure described, by pouring into 4 volumes of ethyl alcohol. Each precipitate was then dried.

Preparation of Salts of the Extracellular Polysaccharide

The desired salt of the polysaccharide was prepared by adding an excess of the appropriate base to an aqueous solution of the polysaccharide. The salt was then recovered by pouring the reaction mixture into 2 volumes of ethyl alcohol. The precipitate was washed with alcohol until the washings were neutral to litmus and then dried in vacuo at room temperature.

Preparation of the Acetate Derivative (Wolfrom and Thompson, 1963)

Method One: (Jones and Painter, 1959)

The polysaccharide was dried in vacuo at room temperature over silica gel for 3 days, then mixed with finely ground anhydrous zinc chloride (1 gram/5 grams polysaccharide),

Glacial acetic acid (100 ml./5 grams polysaccharide) was added and the mixture was heated at 80°C for 4 hours. The mixture was then concentrated to 1/2 volume and poured into a large excess of ice-cold water. The precipitate was collected, washed by decantation and dissolved in chloroform. The chloroform solution was dried with solid Na_2SO_4, filtered through decolorizing charcoal, agitated with ice-cold N Na_2CO_3 in a Waring Blendor for 3 minutes and poured into a separatory funnel. The chloroform layer was twice more treated as described, then washed with water and dried with Na_2SO_4. The chloroform layer was then poured into Skellysolve B, the resulting precipitate collected, washed with Skellysolve B and air dried.

Method Two: (Carson and Maclay, 1946; Young and Adams, 1965)

The dried polysaccharide (1 gram) was dispersed in formamide (10 ml.) in a flask fitted with a thermometer and separatory funnel and containing a magnetic stirring bar. The mixture was stirred at 45-50°C for 1 hour. Pyridine (9 ml.) was added in small amounts over a 30 minute period with vigorous stirring at 45-50°C and then cooled to 30°C. Acetic anhydride (5.2 ml.) was added in 4 portions over a period of 4 hours, then the mixture was stirred at 30°C for 5 hours and allowed to stand overnight at room temperature. The mixture was then poured into 800 ml. of 2 percent HCl (v/v) containing about 100 grams of chopped ice. The mixture was stirred for an hour and filtered with the aid of vacuum.

The precipitate was washed with cold 0.5 percent HCl (v/v) and 4 times with distilled water and placed in distilled water (1 liter) overnight. The precipitate was recovered by filtration, washed with distilled water and dried *in vacuo* over solid NaOH at room temperature.

For re-esterification the material was placed in a flask containing pyridine (25 ml.) and acetic anhydride (4 ml.) and allowed to stand at room temperature for 5 days. The ester was isolated by pouring the mixture into Skellysolve B (200 ml.). The precipitate was filtered, washed several times with petroleum ether, suspended in 1 percent HCl (v/v) and stirred for several hours at room temperature. The precipitate was recovered by filtration and washed with distilled water until free of chloride and dried as previously described. The precipitate was suspended in dioxane and then poured into Skellysolve B (3 volumes). The precipitate was filtered, washed with petroleum ether and dried *in vacuo* at room temperature.

Disc Electrophoresis Techniques (Clark, 1964)

Stock Solutions

Solution A

Acrylamide (Eastman # 5521)	30.0 grams
N, N'-Methylenebisacrylamide (Eastman # 8383)	1.0 gram
Distilled water	123.0 milliliters

Solution B

N_4-Tetramethylethylenediamine

(Eastman # 8178)	0.28 % v/v

Solution C

Glycine	29.0 grams
Tris(hydroxymethyl)amino methane	6.0 grams
Distilled water	980.0 milliliters

Solution D

Ammonium persulfate	0.14 % w/v

The stock solutions were stored in 4 ounce brown polyethylene bottles and were mixed in the ratio of 2 parts A, 1 part each of B and C and 4 parts D just before use. This ratio gave a gel with a 5 percent acrylamide concentration.

Electrolyte Solution

Glycine	29.0 grams
Tris(hydroxymethyl)amino methane	6.0 grams
1 N HCl	5.0 milliliters
Distilled water	975.0 milliliters

The final pH should be 8.1; therefore, if necessary, the amount of acid can be adjusted to provide the correct pH. The stock solution was stored in the refrigerator in a brown polyethylene bottle. The solution was diluted 1:10 with distilled water just prior to use.

Procedures

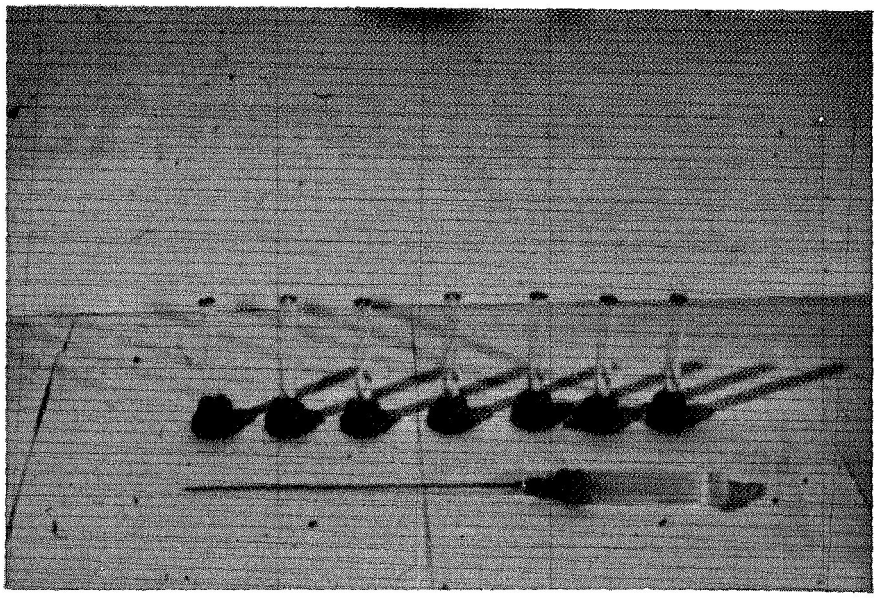
The glass columns (6.5 cm. long) were cut from glass tubing with an inside diameter of 0.6 cm. The ends were not fire polished since this had a tendency to make the ends smaller in diameter and hindered the removal of the gels. The ends were filed where necessary to give a smooth, even

end. The column holders consisted of serum stoppers which were cut off such that a rim of about 3/8 in. remained. The then inverted serum stoppers served both as a base for the glass columns and as a support for the columns (Figure 3).

Before use, the glass columns were washed with a detergent and finely bristled brush, then rinsed thoroughly with tap water, and several times with distilled water. The columns were drained against absorbent paper and placed in a coating solution (Canalco #3-411) for at least 15 minutes after which they were allowed to air dry.

The required amount of gel solution was prepared immediately before use and 1.5 ml. were added to each column inserted in the column holder. Immediately, thereafter, the gel solution was carefully layered with distilled water (about 5 mm.) using a Pasteur pipette. The water served to exclude the air so that chemical polymerization could occur and to prevent the formation of a curved surface on the gel. The polymerization occurred in about 30 minutes during which time it was important not to disturb the columns. Before proceeding further, the water was shaken out of the columns. It was found that a supply of gel columns could be prepared to this point and stored, layered with water, in the refrigerator for several days without noticable changes in the electrophoretic properties.

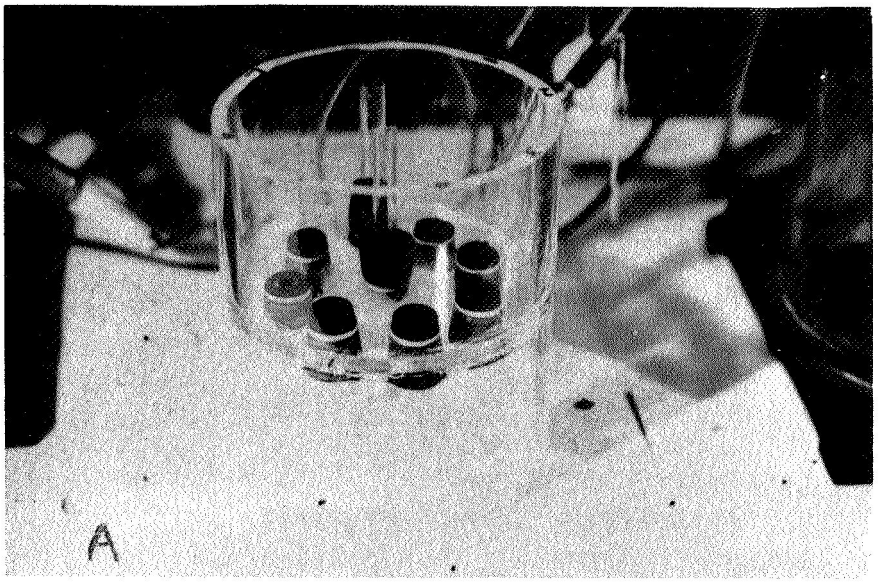
Figure 3. Glass Columns and Column Supports Employed in Disc Electrophoresis.

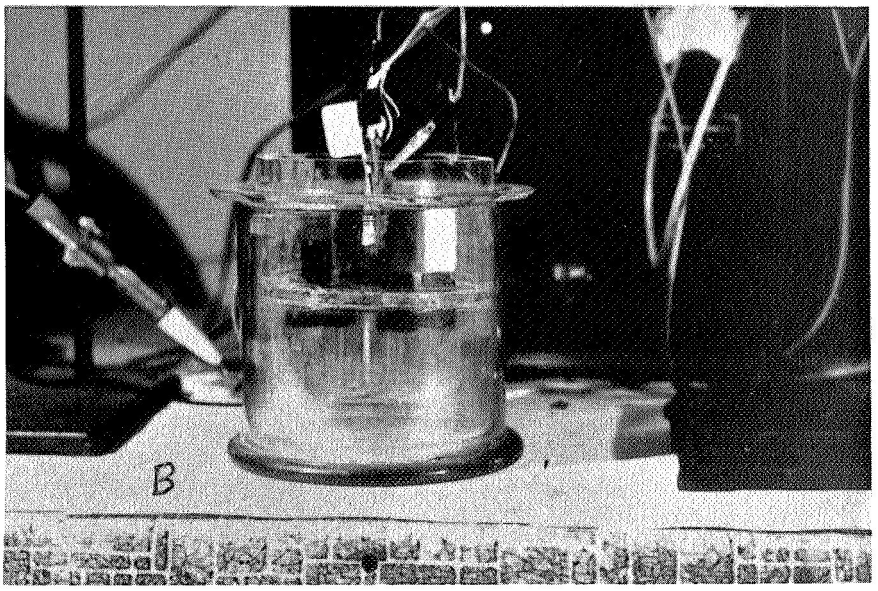


The polysaccharide sample was added to an equal volume of 10 percent sucrose and 0.2 ml. was added to each of the prepared columns. The sucrose was employed to increase the density of the sample such that a minimum of mixing would occur when the column was completed. The columns were completed by adding, very carefully, a quantity of freshly prepared gel solution sufficient to fill the column and allow an excess on the top. The columns were then sealed by placing a very small square of plastic wrap on the top of each column without the formation of air bubbles. If bubbles formed, it was necessary to remove the wrap, add more gel solution and place a fresh piece of wrap on the column. The columns must be sealed within 10 minutes of preparing and adding the gel solution. Polymerization occurred in about 30 minutes and it was important that the columns not be distrubed during this time.

The electrophoresis chamber consisted of a plastic cylinder (about 5 in. in diameter and about 10 in. high) with a lucite partition dividing the cylinder into 2 equal parts (Figure 4, A). The partition had 8 holes drilled around the outer perimeter and a hole in the center. The holes were of the size to accommodate a number 2 rubber stopper. The completed columns were inserted into number 2 rubber stoppers such that the sample end was even with the smaller end of the stopper. The columns were then placed in the partition such that the sample end extended into the

Figure 4. Disc Electrophoresis Chamber (A) and Chamber Holder (B) Employed in Disc Electrophoresis.





upper chamber. The upper part of the cylinder chamber served as the upper buffer reservoir. The cylinder was set into a glass vessel which served as the lower buffer reservoir (Figure 4, B). The anode was inserted into the lower compartment via the center opening which contained a rubber stopper fitted with glass tubing which served both to support and to protect the electrode. The cathode was wrapped around the glass tubing in the center of the upper compartment. Five hundred milliliters of the diluted electrolyte solution were poured into each compartment. Electrophoresis was conducted at a current of either 4 or 5 milliamperes per gel with a maximum of 7 gels/run for the desired length of time using a high voltage power supply (Figure 5). The eighth hole was equipped with a rubber stopper into which a thermometer could be inserted for monitoring the temperature changes if desired.

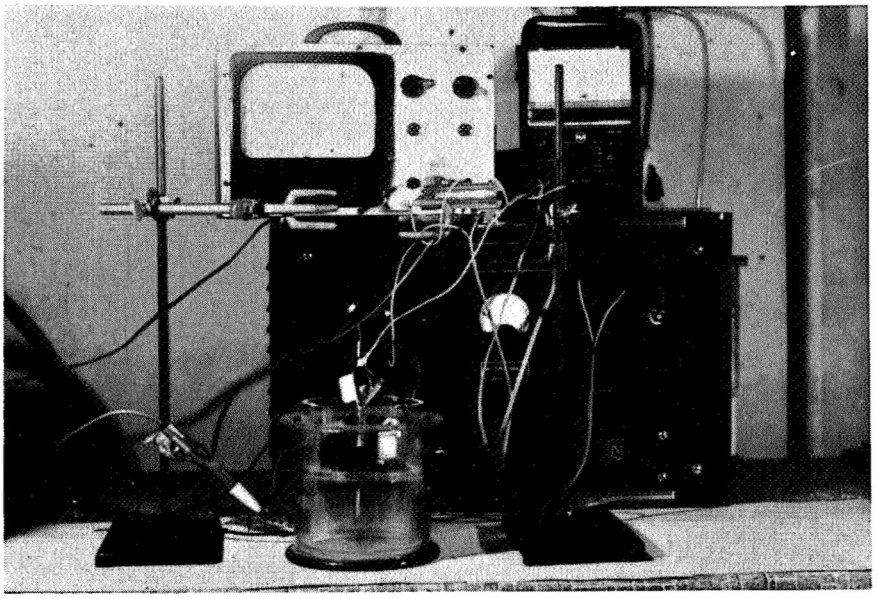
Procedures for Staining the Gels

After electrophoresis the gels were carefully removed from the columns with the aid of a long 19 gauge needle (refer to Figure 3) which was used to rim the gels and to inject water during the rimming process to facilitate the removal of the gels. The gels were then placed in individual test tubes and rinsed with distilled water, prior to staining, in order to remove any residual sample.

Polysaccharide Staining

For polysaccharide staining the gels were placed in an

Figure 5. High Voltage Power Supply Utilized in Disc Electrophoresis.



excess of the oxidizing solution (1 percent periodic acid in 3 percent aqueous acetic acid, Clark, 1964) for 1 hour after which the gels were rinsed with and leached with distilled water for 1 hour. Then the gels were submerged in Schiff's reagent (Clark, 1964) in the refrigerator until the bands appeared--about 2 hours. The gels were rinsed with, and stored in, a solution consisting of 1 percent aqueous sodium metabisulfite. A modified staining technique for polysaccharides has been described by Stewart-Tull (1965).

Protein Staining

For protein staining the gels were placed in an amido black reagent (1 percent amido black in 7 percent aqueous acetic acid, Davis, 1964) for at least an hour, preferably for 3 or 4 hours. The gels were rinsed with and then leached with a 7 percent aqueous acetic acid solution until no further stain was removed. Alternatively, the stain was removed electrophoretically using the acetic acid solution as the electrolyte and a current of 12 mA/gel for about 15 minutes.

Infra-red Spectrophotometric Techniques (Barker, Bourne and Whiffen, 1956; Willard, Merritt and Dean, 1965; Tipson, 1968; Spedding, 1964; Kuhn, 1950)

Infra-red analyses of the polysaccharides and fractions were performed by applying aqueous solutions to the surfaces of silver chloride windows. The solutions were dried with an infra-red lamp. All measurements were made with a Perkin-Elmer Model 137 Infra-red Spectrophotometer.

Viscosity Measurements (Daniels, et al., 1962)

The viscosity of the aqueous solutions of the extracellular and the TCA extracted polysaccharide was determined with an Ostwald-Fenske viscosimeter. The time was determined with a Meylan stopwatch calibrated to 0.5 second. All temperature measurements were made using the Fahrenheit scale. The specific gravity of the solutions was determined with a hydrometer and the density obtained by converting with the aid of tables (ASTM-IP,1952).

Film Formation (Whistler and Smart, 1953)

A film of the polysaccharide was obtained by casting a thin sheet of an aqueous solution of the extracellular polysaccharide on a glass plate and allowing it to dry at room temperature.

Optical Activity (Daniels, et al., 1962)

The optical rotation of the polysaccharide was determined on aqueous solutions of the natural acidic form and the sodium salt. The determinations were made on a saccharimeter, using a 100 mm. tube at room temperature. The readings were in angular degrees.

EXPERIMENTAL RESULTS AND DISCUSSION

Extracellular Polysaccharide Production in Relation to Population Growth

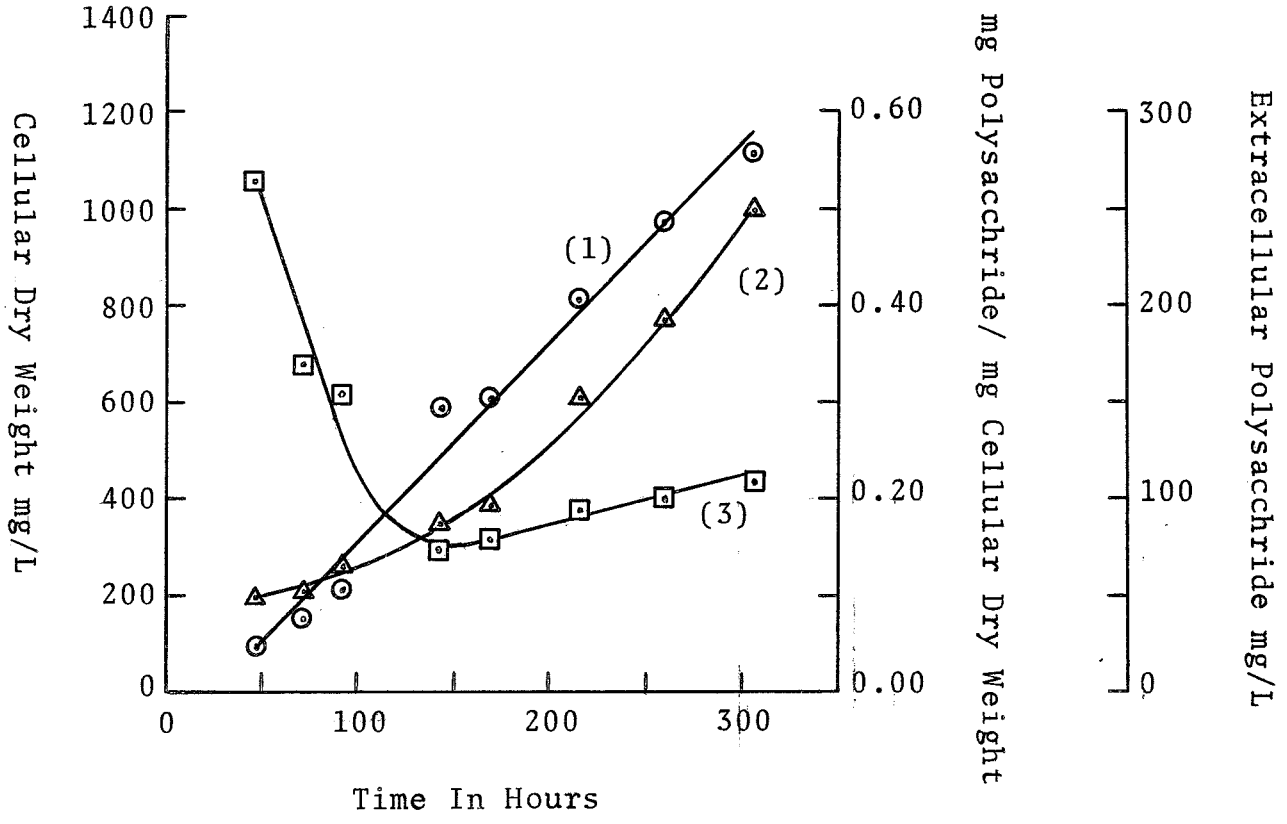
To determine polysaccharide production in relation to population growth in *A. flos-aquae* A-37, the organism was grown in the basal medium plus KNO_3 (1 gram/liter). Duplicate large cultures were set up and samples were aseptically removed at various times for the determination of cellular dry weight and polysaccharide production. The amount of polysaccharide was determined by the phenol-sulfuric acid method utilizing the supernatant obtained after filtering the culture sample onto the asbestos mat in the tared Gooch crucible. Each determination was done in duplicate and then averaged for the two cultures. These results are shown in Figure 6. The population growth is shown as a linear regression (curve 1). Analysis of variance indicated that the regression was highly significant. At the final sampling, after 307 hours or almost 13 days of growth, the polysaccharide production had reached 250 mg./l. (curve 2) and the population growth as expressed in cellular dry weight (curve 1) had reached 1.12 gm./l. It may be observed that even after almost 13 days of cultivation, the cultures have not entered a stationary phase of growth. The polysaccharide production appears to be directly related to the cellular and population growth since production occurs throughout

Figure 6. Extracellular Polysaccharide Production and Increase in Cell Mass Expressed as a Function of Time.

(1) Cellular Dry Weight. $\hat{Y} = 4.159\ X - 106.6$

(2) Extracellular Polysaccharide.

(3) Ratio of Polysaccharide to Dry Weight.



the growth period. The ratio of polysaccharide produced to the cellular dry weight is presented in curve 3. The ratio is the greatest at the beginning of the sampling period probably because the cultures are coming out of the lag phase. As the population increases the ratio drops to its lowest value at about 6 days. The ratio begins to increase about the seventh day and a definite upward trend is apparent at the termination of cultivation. It may be speculated that the ratio would reach a maximum during the stationary phase.

The Effect of Various Nitrogen Sources on Extracellular Polysaccharide Production

The effect of various nitrogen sources on the production of extracellular polysaccharide by A. flos-aquae A-37 was determined by growing the organism in the basal medium plus a variety of nitrogen sources added to provide the same amount of nitrogen as that in 1 gram of KNO_3. The inoculum for this experiment was grown with elemental nitrogen as the nitrogen source. The cultures were allowed to grow for 5 days in an air atmosphere, after which they were assayed for the amount of polysaccharide present in the spent medium using the phenol-sulfuric acid method. The results of these studies (Table 1) are expressed as the quantity of polysaccharide produced, population growth and pH. The statistical analysis of the polysaccharide data is found in Table 2. There was a highly significant difference among the quantities of polysaccharide produced. This observation is of

Table 1. The Effect of Various Nitrogen Sources on the Production of the Extracellular Polysaccharide by A. flos-aquae A-37.

Nitrogen Source	Concentration	Polysaccharide	Final pH	Cells/ml. x 10^7
	g/l	mg./100 ml.		
$Mg(NO_3)_2$	1.27	10.70[1]	6.9[2,3]	4.5[1]
KNO_3	1.00	9.10	7.0	3.3
$NaNO_3$	0.84	8.20	7.2	4.0
NH_4NO_3	0.39	6.67	5.8	2.7
NH_4Cl	0.53	4.77	5.4	2.4
$Ca(NO_3)_2$	0.81	4.47	7.1	0.4
$NH_4H_2PO_4$	1.13	3.57	6.5	2.5
N_2 (control)	----	1.67	6.6	2.3

[1]Each value is the average of three replications with duplicate determinations done on each replication.

[2]The initial pH was 8.0.

[3]Each value is the mean of two determinations.

Table 2. Statistical Analysis of the Polysaccharide Data Shown in Table 1.

ANALYSIS OF VARIANCE[1]				
Source	df	SS	MS	F
Treatments	7	196.19	28.03	12.19**[2]
Replications	2	6.22	3.11	1.35 n.s.
Error	14	32.21	2.30	
Total	23	234.62		

$s_{\bar{x}} = 0.876$

[1]Steel and Torrie, 1960.

[2]Highly significant at the 0.01 level of probability.

particular interest when considered with respect to the population growth estimated by cell counts. The largest polysaccharide yield occurred in the culture which had the greatest population. This quantity could have been due, in part, to the stimulatory effect of the magnesium on photosynthesis. Little difference existed among the populations of the cultures grown on NH_4NO_3, NH_4Cl, $NH_4H_2PO_4$ and N_2; however, large differences were observed in the quantities of polysaccharide produced. The lowest yield of polysaccharide was obtained when the alga was fixing nitrogen which may indicate that the energy and foundation materials derived from photosynthesis and photophosphorylation were being directed toward cellular or population growth rather than the manufacture of excess sugars. It may be assumed that more energy would be required to convert elemental nitrogen into a form suitable for utilization by the cellular machinery. This would automatically reduce the energy and raw materials available for polysaccharide production. An interesting phenomenon is observed in the data from the culture supplied with $Ca(NO_3)_2$. This culture exhibited the least amount of growth; however, the quantity of polysaccharide produced was greater than that produced with either $NH_4H_2PO_4$ or N_2. It may be assumed that either the calcium exerted an inhibitory effect on growth or that the alga was unable to utilize this nitrogen source.

The pH of the cultures probably exerted some influence on the quantity of the polysaccharide produced. Aside from the changes due to metabolism, the pH of the control (N_2) was influenced solely by the formation of carbonic acid from the carbon dioxide in the air supply, whereas the pH of the other cultures was additionally influenced by the nitrogen source as it was utilized.

Solubility Tests

Solubility tests were conducted on the extracellular polysaccharide with the expectation of running partition studies via countercurrent extraction (Craig, 1962). The dried polysaccharide was placed in 5 ml. of the solvent and allowed to equilibrate for several hours. Changes in the external appearance such as swelling were noted. Those solvents which had effected a change in the external appearance were investigated further with chemical tests using phenol and sulfuric acid. The water-immiscible organic solvents were extracted with water and the aqueous layer was then tested for the presence of the polysaccharide. Controls were run for each solvent tested. The solvents tested and the results are presented in Table 3. These results indicated that the polysaccharide was insoluble in all except aqueous solutions and some water-miscible solvents. The sodium and potassium salts of the polysaccharide were found to behave in a similar manner.

Table 3. Solvents Tested for Use as a Polysaccharide Solvent.

Water-Immiscible	Water-Miscible[1]
Chloroform	Dioxane
Ethyl acetate	Pyridine
Carbon tetrachloride	Acetone
Toulene	Propyl alcohol
Ether	Ethylene glycol +[2]
Cyclohexane	Formamide +
2,2,4 Trimethyl pentane	Methyl alcohol
n-Butanol	Acetic acid +
Phenol	Ammonium hydroxide +
Aniline	
Carbon disulfide	
Tertiary amyl alcohol	
Isoamyl alcohol	Buffers
Amyl acetate	
Benzene	Phosphate M/15 +
Benzyl acetate	Acetic acid-sodium acetate +
Butyl acetate	Borate +
Benzaldehyde	
Nitrobenzene	
Hexane	
Decane	
Isobutyl alcohol	
Pentane	
n-Butyraldehyde	

[1]All of these solvents are polar. They are listed in order of increasing polarity.

[2]This symbol indicates those solvents and solutions in which the polysaccharide was soluble to some extent.

The barium salt was insoluble in aqueous solutions with the exception of the acidic ones, e. g. dilute HCl and dilute H_2SO_4.

In an attempt to overcome this insolubility, the extracellular polysaccharide was treated in order to prepare an acetate derivative using the methods previously described. The resulting derivative was found to be insoluble in chloroform, Skellysolve B, carbon disulfide and other solvents, with the exception of dioxane in which is was only very slightly soluble. This observation agreed with that of Bishop, et al. (1954) for the polysaccharide produced by Anabaena cylindrica.

The formation of a water-insoluble quaternary ammonium salt of the polysaccharide was accomplished by the addition of Cetyltrimethylammonium bromide (Cetavlon) (2 percent) to an aqueous solution of the polysaccharide (Scott, 1955, 1960; Stacey and Barker, 1960). The resulting precipitate was washed with distilled water and dried. This salt was found to be soluble only in dilute solutions of NaCl (e. g. 0.25 and 1 M) and dilute acetic acid.

Thus, the preparation of derivatives and salts did not result in the production of a more soluble product than the natural one. Therefore, the consideration for partitioning the polysaccharide between two immiscible solvents was rejected.

The techniques developed by Albertsson (1960) for the partition of cell particles and macromolecules in aqueous

polymer solutions were considered. These techniques are based on the fact that certain polymer solutions form distinct layers at discrete concentrations. The systems investigated were dextran-methylcellulose, dextran-polyethylene glycol and potassium phosphate-polyethylene glycol. The first system was rejected because the time required for the separation of the two phases was on the order of 12 hours. At room temperature, this allowed for the growth of microorganisms which contaminated the samples. The second system had a settling time of about 30 minutes; however, the dextran interfered with all tests for the detection of the polysaccharide. This system was also rejected. The third system had a settling time of about 15 minutes; however, the solubility of the polysaccharide was so slight that it could be neither detected nor recovered from the phases. Thus, all attempts to partition the polysaccharide were unsuccessful.

Studies on the Polysaccharide Constituents

A series of studies on the extracellular polysaccharide obtained under various conditions was conducted to elucidate the monomers of the polysaccharide.

Two Dimensional Paper Chromatography

Two dimensional paper chromatography of the extracellular polysaccharide hydrolyzates was employed to reveal the constituents. The results of this study and accompanying sugar standards are given in Table 4.

Table 4. R_f values, $R_{glucose}$ values and Color Reactions of Standard Sugars and Extracellular Polysaccharide Constituents.[1]

Compound	Solvent 1[2]	Solvent 2[3]	Color Reaction with Reagent B
	R_f[4]	$R_{glucose}$[4]	
	Standards		
Uronic acids[5]	11	0.09	yellow-brown
Glucose	43	1.00	yellow
Galactose	50	0.86	yellow
Mannose	52	1.31	yellow
Arabinose	61	1.34	pink
Xylose	53	1.64	pink
Fucose	71	1.74	yellow
Ribose	70	1.91	pink
Rhamnose	70	2.35	yellow
Glucuronic acid lactone	58	2.62	yellow-brown
Lactose	35	0.17	yellow
2-Deoxyribose	80	----	pink (turns gray)
Lyxose	50	1.76	pink
Unknowns (polysaccharide compounds)			
Uronic acids	10	0.06	yellow-brown
Glucose	42	1.00	yellow
Galactose	45	0.75	yellow
Mannose	49	1.20	yellow
Arabinose	59	1.23	pink
Xylose	52	1.53	pink
Fucose	69	1.59	yellow
Ribose	68	1.75	pink
Rhamnose	71	2.26	yellow

1 Values were obtained with paper chromatography.

2 Phenol-water saturated

3 Butanol-pyridine-water

4 These values are the averages for several chromatograms.

5 In most instances there was no separation of glucuronic and galacturonic acids.

The comparison of R_f and $R_{glucose}$ values and the color reactions allowed the tentative identification of the components as uronic acids, galactose, glucose, mannose, xylose, arabinose, ribose, fucose and rhamnose. Tests with the ninhydrin spray revealed no amino acids.

The completion of this study and the tabulation of the data brought out the fact that an obvious discrepancy existed between these results and those previously reported for the extracellular polysaccharide produced by A. flos-aquae A-37 by Moore and Tischer (1965). The components reported by them were glucuronic acid, glucose, xylose and ribose.

Several explanations were offered for this discrepancy: (1) the possibility of a mutation since the alga used for these studies (referred to as the subculture) had been subjected previously to studies on nitrogen fixation (Davis, Tischer and Brown, 1966); (2) the possibility of a modification as a result of a change in the growth medium, i. e. Knop's algal medium was employed by Moore and Tischer (1965); and (3) the possibility that these components were present only when the alga was grown under the conditions imposed by the author.

The first possibility was investigated by having Tischer set up the original isolate of A. flos-aquae A-37 in growth medium for cultivation after which the cultures were taken

by the author and the polysaccharide extracted and examined. These results are found in Table 5. From these results, it may be seen that the polysaccharides contained the same components. This indicated that no mutation had occurred or that if it had, the original isolate had mutated likewise which would seem to have a probability approaching zero.

The second possibility was investigated by growing the test alga in the Knop's medium which was employed by Moore and Tischer (1965). These results are presented in Table 6. Since the same components were present in both of the polysaccharides, it is apparent that no modification was induced by the change in the growth medium.

The last possibility to be considered was investigated by obtaining some spent medium from Mr. C. D. Bostwick[5] who was also working with A. flos-aquae A-37. The polysaccharide was extracted and examined. Table 7 contains these results which show that there was no difference in the chromatographic pattern of the two polysaccharides.

The consideration of these possibilities still left the discrepancy between the two reports unresolved. The only other possibility which can be offered can not be investigated because the conditions under which this work was conducted can not be replicated. It seems reasonable

[5]Graduate student, Department of Microbiology, Mississippi State University.

Table 5. Polysaccharide Constituents in the Polysaccharides Produced by the Original Isolate and the Subculture of A. flos-aquae A-37.[1]

Compound	R_f[2]		$R_{glucose}$[3]	
	OI[4]	SC	OI	SC
Uronic acids	10	6	0.1	0.1
Glucose	47	44	1.00	1.00
Galactose	52	49	0.76	0.76
Mannose	53	51	1.30	1.27
Arabinose	63	61	1.34	1.30
Xylose	55	53	1.63	1.56
Fucose	73	71	1.79	1.63
Ribose	72	69	1.90	1.79
Rhamnose	73	71	2.37	2.22

[1]The constituents were resolved with paper chromatography.

[2]Phenol-water saturated solvent

[3]Butanol-pyridine-water solvent

[4]The designation OI refers to the original isolate and SC refers to the subculture.

Table 6. Polysaccharide Constituents in the Polysaccharide Produced by *A. flos-aquae* A-37 When Grown on Knop's Medium.[1]

Compound	R_f[2]	$R_{glucose}$[3]
Uronic acids	6	0.10
Glucose	44	1.00
Galactose	50	0.87
Mannose	53	1.39
Arabinose	62	1.26
Xylose	53	1.73
Fucose	71	1.69
Ribose	69	1.88
Rhamnose	72	2.53

[1]The constituents were resolved by paper chromatography.

[2]Phenol-water saturated solvent

[3]Butanol-pyridine-water solvent

Table 7. Polysaccharide Constituents in the Polysaccharide Produced by *A. flos-aquae* A-37 Cultivated by Mr. C. D. Bostwick.[1]

Compound	R_f[2]	$R_{glucose}$[3]
Uronic acids	6	0.10
Glucose	44	1.00
Galactose	49	0.76
Mannose	51	1.27
Arabinose	61	1.30
Xylose	53	1.56
Fucose	71	1.63
Ribose	69	1.79
Rhamnose	71	2.22

[1]The constituents were resolved by paper chromatography.

[2]Phenol-water saturated solvent

[3]Butanol-pyridine-water solvent

to assume that, somehow, the alga with which they worked was a different variety which was selected by some random chance from the original isolate of A. flos-aquae A-37.

Confirmation of the Identity of the Unknowns

The identity of the sugar spots was confirmed by running the eluted sample along with the suspected sugar standard using thin-layer chromatographic techniques. It was possible to confirm all unknowns with the exception of the uronic acids. The tentatively identified compounds moved and reacted identically with the authentic sugar standards. It is speculated that the polysaccharide contains both galacturonic and glucuronic acids. Glucuronic acid was previously reported by Moore and Tischer (1965). This speculation is based upon the fact that A. flos-aquae A-37 has been shown to contain the enzyme UDP-D-Galacturonic Acid-4-epimerase which would allow the utilization of either moiety in the manufacture of the polysaccharide (Ankel and Tischer, 1968).

Studies to Determine the Homogeneity of the Polysaccharide

The fractionation effected according to method one resulted in 2 fractions, one very slight and the other very abundant. The composition of the 2 fractions was found to be chromatographically the same. The results were the same as those found in the preceding tables.

The fractionation procedure according to method two

resulted in the formation of 3 fractions, one due to the formation of a cupric complex and the others as a result of alcohol precipitation. The last fraction was very small. All of these were analyzed with chromatographic techniques and were found to contain the same components.

An attempt was made to fractionate any neutral polysaccharides which might be present. This was accomplished by first removing the acidic polysaccharides which were known to be present, by the addition of 10 percent Cetavlon. The precipitate was removed by centrifugation and the supernatant treated to recover any neutral polysaccharides. The supernatant was mixed with an equal volume of 1 percent borate buffer, pH 8.5; however, no precipitate was formed. The addition of more Cetavlon and buffer did not effect any precipitate. The procedure was repeated on a fresh sample, this time using 1 percent borate buffer, pH 10. The results were again negative.

The method of Young and Adams (1965) for the fractionation of polysaccharides was employed. The only fraction obtained consisted of acidic polysaccharides. The constituents of this fraction were as previously given for the polysaccharide. The complex formed with Cetavlon did not affect the chromatographic pattern.

The conclusions drawn from this series of studies were that (1) the polysaccharide was composed of several

like polymers, differing perhaps in molecular weight and (2) the polysaccharide was composed of acidic polymers. Therefore, it was assumed that the polysaccharide could be regarded as being homogeneous on a macroscale.

Studies to Determine the Effect of Aqueous Solutions of Various Inorganic and Organic Compounds on the Polysaccharide

An aqueous solution of the polysaccharide was treated with aqueous solutions of a variety of inorganic and organic compounds to determine if these compounds had any effect on the polysaccharide. These tests revealed that some compounds effected precipitation, whereas others effected a clarification of the aqueous solution of the polysaccharide. Those compounds which brought about the precipitation of the polysaccharide, usually with the addition of only a few drops of the reagent, are presented in Table 8. Most of these reagents reacted with the polysaccharide to form a water-insoluble precipitate, i. e. a precipitate which remained insoluble when placed in distilled water. The exceptions to this were the precipitates formed with NaOH, KOH and ethyl alcohol. This precipitation reaction was probably due to the presence of the free carboxyl group in the uronic acid monomer on the molecule. This reaction, in most cases, resulted in the formation of a salt of the polysaccharide. Many of these reagents are used in the identification of plant gums and mucilages (Smith and Montgomery, 1959).

Table 8. Compounds Which Effect Precipitation of the Extracellular Polysaccharide Produced by *A. flos-aquae* A-37.

Compound	Concentration of solution employed
NaOH	2.5 N
KOH	1.0 N
Cetavlon (Cetyltrimethylammonium bromide)	2.0 %
$Ca(OH)_2$	Saturated
$Ba(OH)_2$	Saturated
Lead acetate (neutral)	20.0 %
Lead acetate (basic)	20.0 %
$CuSO_4$	7.0 %
$MgSO_4$	2.0 %
$FeSO_4$	2.0 %
$MnCl_2$	2.0 %
$ZnCl_2$	2.0 %
$Al_2(SO_4)_3$	2.0 %
$FeCl_3$	2.0 %
Ferric citrate	Saturated
Ethyl alcohol	99.5 %

Another group of reagents was found to bring about a clarification of the aqueous solution of the polysaccharide. These reagents are found in Table 9. The mode of action of these agents has not been determined, but some of the agents are well known chelating agents.

Studies Employing Disc Electrophoresis Techniques

The initial disc electrophoresis studies were conducted using stock solutions obtained from Canalco, Rockville, Maryland, following the procedure described in the phamplet accompanying the solutions. Their procedure was that originally described by Davis (1964). This procedure was found to be unsatisfactory because of the difficulty in getting the sample layer to polymerize with the polysaccharide sample.

After investigating several alternative procedures, the procedure described by Clark (1964) was employed, with minor modifications. In this method, the polysaccharide was not required to be polymerized in the column. The major modification in this procedure consisted of preparing a gel layer on top of the sample rather than using the cotton plug which was found to be unsatisfactory.

In order to make sure that other substances would not interfere with the electrophoresis, tests were run substituting distilled water for the sample in order to test the sucrose, since this was employed to increase the density of

Table 9. Compounds Which Effect Clarification of the Extracellular Polysaccharide Produced by *A. flos-aquae* A-37.

Compound	Concentration of solution employed
Mg citrate	2.0 %
H_3PO_4	2.0 %
Citric acid	5.0 %
EDTA	Saturated
$(NH_4)_2SO_4$	Saturated
$(NH_4)_2SO_4$	30.0 %
$(NH_4)_2SO_4$	10.0 %

the sample solution. There was no indication of a reaction or interference with the control. During the early trial runs a tracking dye composed of 0.001 percent Bromphenol Blue (5 ml./liter of electrolyte) was utilized to follow the rate of electrophoresis.

The polysaccharide sample was prepared by dissolving a portion of either dried or freshly extracted polysaccharide in a sufficient amount of distilled water to effect solution. Any insoluble material was removed by either centrifugation or filtration. It was found that the quantity of polysaccharide in the sample was not critical. The pores of the gel allowed only a limited quantity to enter; however, it was necessary to have an amount sufficient to be detected after staining.

In order to determine if any protein was associated with the polysaccharide, the electrophoresized samples were stained with amido black 10 B. After destaining, no bands were present to indicate the presence of protein.

The polysaccharide hydrolyzate as well as the individual sugar standards were subjected to electrophoresis to determine if any of the individual components would produce a reaction. All of the individual components were subjected to electrophoresis, separately and combined. The results indicated that none of them reacted to the procedure.

Figure 7 illustrates the typical pattern for the changes in voltage and temperature during an electrophoresis run at a constant current. The temperature differential between the chambers becomes greater as the time of electrophoresis is increased. The temperature was higher in the lower chamber because the major portion of the column extends into that chamber.

In order to determine the effect of electrophoresis on the sodium salt of the polysaccharide, the sample was subjected to electrophoresis at a constant current of 5 mA/gel for increasing lengths of time. Figure 8 illustrates the results. The distance was measured in cm. from the sample origin. It was found that only 1 band was present, regardless of the length of time for electrophoresis, and that this band moved proportionally to the time. It may also be noted that as the time increased the band became more curved.

Figure 9 illustrates the extracellular (acidic) polysaccharide run for 2 time periods at a constant current of 5 mA/gel. It may be noted here again that only 1 band was present. The characteristics of the band were the same as those noted for the sodium salt.

A series of runs was set up to determine if any component in the polysaccharide might move in the reverse direction, i. e. toward the cathode. In the first tests,

Figure 7. Voltage and Temperature Changes During Disc Electrophoresis at a Constant Current of 4 mA/gel.

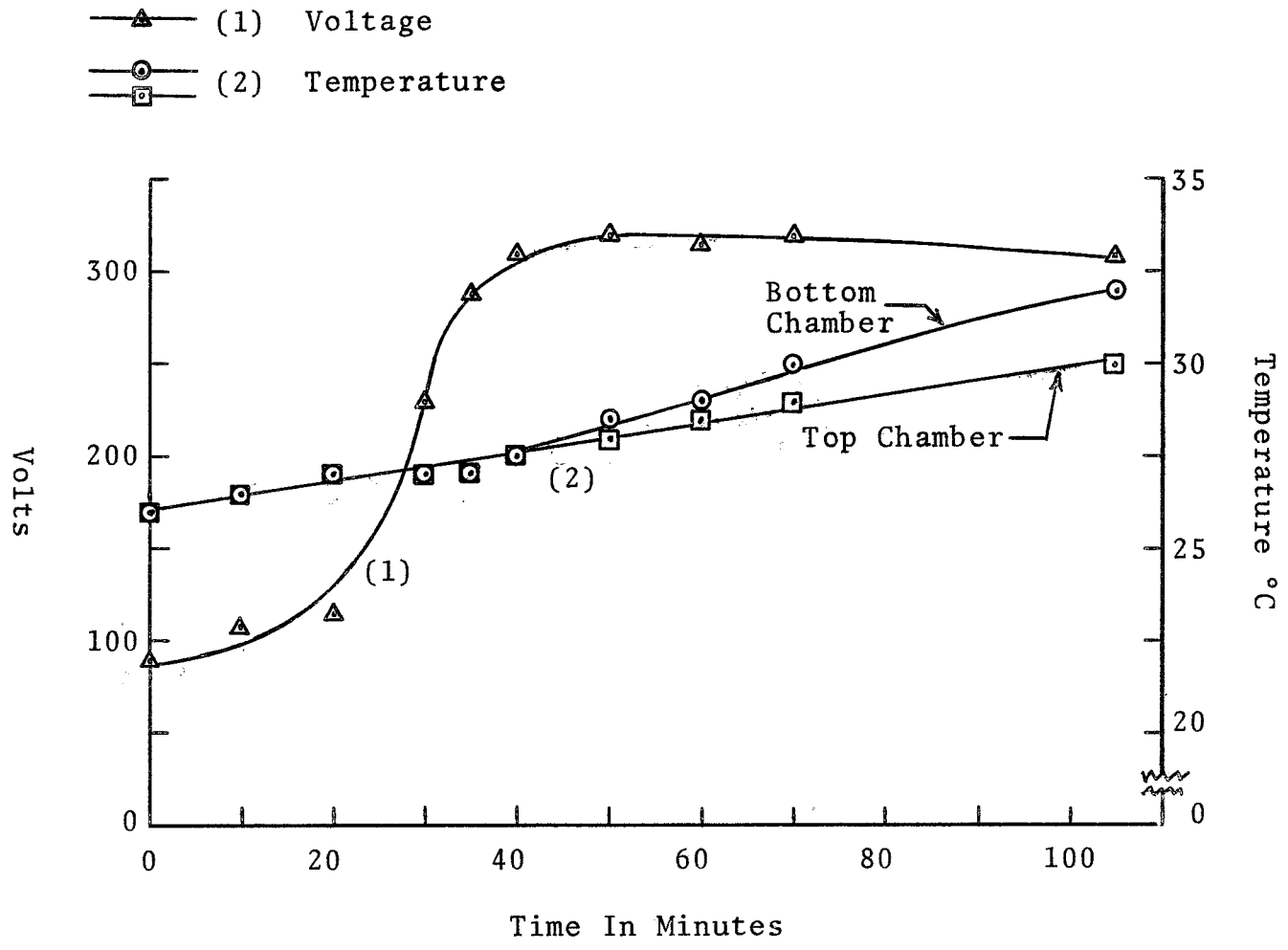


Figure 8. Disc Electrophoresis of the Sodium Salt Polysaccharide at a Constant Current of 5 mA/gel.

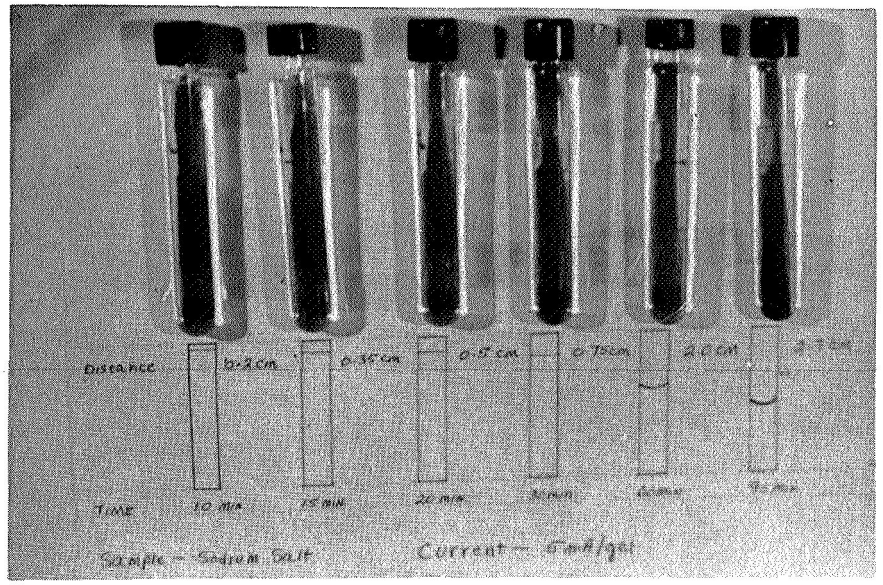
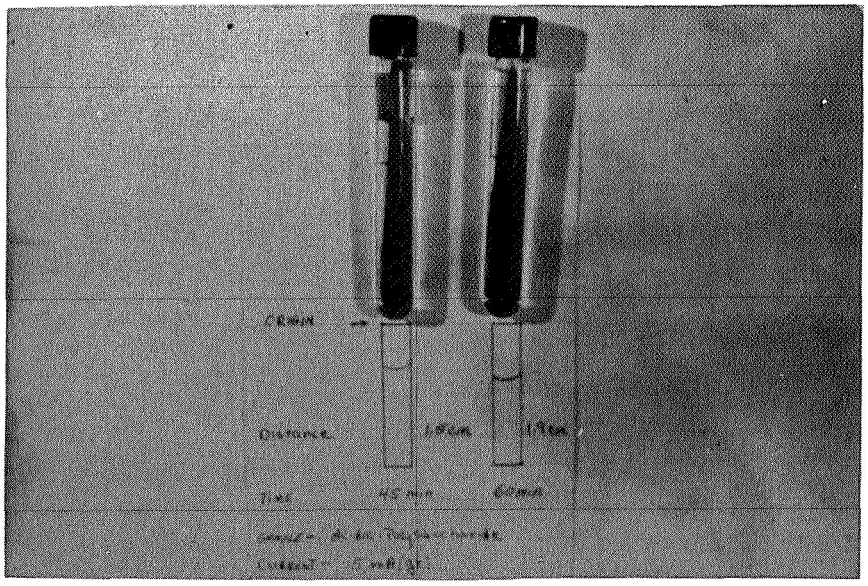


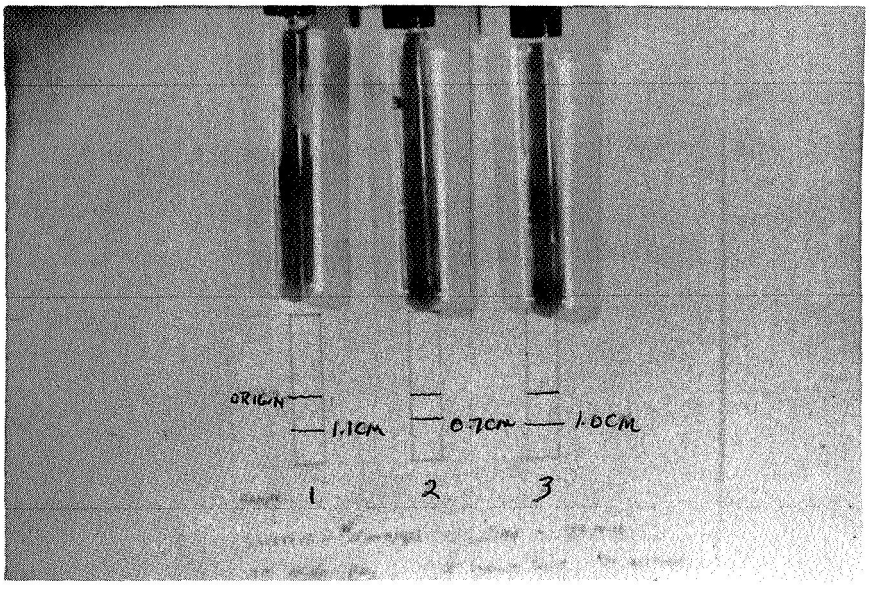
Figure 9. Disc Electrophoresis of the Extracellular (acidic) Polysaccharide at a Constant Current of 5 mA/gel.



all samples were set up as usual and the chamber prepared. After all preparations were completed, the electrodes were reversed such that the cathode was in the lower chamber. The duration of the run was 30 minutes with the samples being 5 polysaccharide and 2 water controls. After staining, the gels were examined and there were no bands whatsoever in any of the gels. In a second series of tests, the gel columns were modified such that the sample was placed in the middle of the gel column rather than near one end. The samples run were: the extracellular (acidic) polysaccharide (no. 1); the sodium salt (no. 2); and a mixture of the two (no. 3). (Figure 10). There was no band present in the upper portion of the gels indicating that the components moved only toward tha anode. In the lower gel of No. 3 an interesting phenomenon was observed. The combined sample contained a band intermediate between the two individual samples, rather than two distinct bands. This indicated that the difference between the extracellular (acidic) polysaccharide and the sodium salt of the polysaccharide was so slight that the samples were able to merge and to move as one sample.

In another series of studies, 7 samples of freshly extracted extracellular polysaccharide were subjected to electrophoresis at a current of 5 mA/gel for 60 minutes after which the samples were removed with a syringe and pooled for another run. The removed samples were run at

Figure 10. Disc Electrophoresis of the Extracellular (acidic) and Sodium Salt Polysaccharides at a Constant Current of 5 mA/gel.



a current of 5 mAlgel for 60 minutes. The results of this series of tests are shown in Table 10. It may be seen that in the second run the band did not travel as far as in the first run. In fact, it moved only half the distance traveled by the sodium salt. However, when the two samples were combined only 1 band was apparent indicating that no difference existed between the two samples. Therefore, it may be concluded that the removed samples contained the same polysaccharide as previously noted.

In another study, the fractions of the extracellular polysaccharide obtained by alcohol fractionation (method one) were examined electrophoretically. These fractions were designated F-1 and F-2 and were run with the sodium salt as the control. A sample containing both fractions and the sodium salt was also run. These results are found in Table 11. It may be seen that there was no difference in the distance traveled by the two fractions. Here again is observed the phenonomenon that the extracellular polysaccharide fractions and the sodium salt merged to move as one substance.

A study was done to determine the effect of stopping electrophoresis of the extracellular polysaccharide for a short period of time and then resuming electrophoresis. The results are found in Table 12. It is interesting to note that a second band appeared during the second run.

Table 10. Disc Electrophoresis[1] Studies on the Extracellular Polysaccharide.

Run 1		Run 2	
Sample	Distance	Sample	Distance
	cm		cm
EP-1[2]	2.0	EP-1	0.8
EP-2	2.0	EP-2	0.8
EP-3	2.1	EP-3	0.8
EP-4	2.1	Na salt-1	1.6
EP-5	2.1	Na salt-2	1.6
EP-6	2.1	Mixture-1	1.2
EP-7	2.1	Mixture-2	1.1
Mean	2.07		

[1]Electrophoresis was conducted at a current of 5 mA/gel for 60 minutes.

[2]EP refers to the extracellular polysaccharide and the number refers to the particular gel column.

Table 11. Disc Electrophoresis[1] of the Fractions of the Extracellular Polysaccharide.

Sample	Distance cm	Mean
F-1	2.1	2.15
F-1	2.2	
F-2	2.2	2.15
F-2	2.1	
Na salt	1.9	
F-1, F-2, Na salt	2.1	2.15
F-1, F-2, Na salt	2.2	

[1]Electrophoresis was conducted at a current of 5 mA/gel for 60 minutes.

Table 12. Disc Electrophoresis[1] of the Extracellular Polysaccharide When the Current was Interrupted.

Sample	Distance			
	Band 1	Mean	Band 2	Mean
	cm		cm	
F-1	3.6	3.55	1.0	0.95
F-1	3.5		0.9	
F-2	3.5	3.30	1.1	1.05
F-2	3.1		1.0	
F-1, F-2	3.4	3.35	1.0	0.95
F-1, F-2	3.3		0.9	
Na salt	3.1		1.0	

Control EP[2]	Distance
	cm
90 minutes	3.30
30 minutes	1.10

[1]Electrophoresis was conducted at a current of 5 mA/gel for 60 minutes, stopped for 10 minutes and resumed at a current of 5 mA/gel for 30 minutes.

[2]EP refers to the extracellular polysaccharide.

However, it was determined that this was the same substance as that in the first band since it moved at the same rate. This may be seen by comparing the sample data with the control data. It was found that a new band would appear in the gel each time the electrophoresis was interrupted and resumed. The number of bands would be limited, of course, by the quantity of sample present.

The TCA-polysaccharide extracted from the washed cells was subjected to electrophoresis to determine if it possessed the same electrophoretic characteristics as the extracellular polysaccharide which was run as the control. There was no band in the gels which contained the TCA-polysaccharide. The normal band was present in the controls indicating that the procedure was correctly done. These results indicated that this TCA-polysaccharide differed from the extracellular polysaccharide and that it was, probably, a neutral polysaccharide.

The conclusion drawn from the disc electrophoresis studies was that the extracellular polysaccharide produced by A. flos-aquae A-37 was electrophoretically homogeneous. This conclusion was based upon the fact that (1) both of the fractions of the polysaccharide exhibited the same electrophoretic mobility and (2) no electrophoretic difference existed between the extracellular polysaccharide and the sodium salt. This conclusion added further support to the

previous indication that this was a homogeneous substance.

Studies to Compare the TCA-Polysaccharide with the Extracellular (Acidic) Polysaccharide

The TCA-polysaccharide in aqueous solution was tested with several reagents to determine if it reacted in a manner comparable to the extracellular (acidic) polysaccharide. These results are shown in Table 13. It may be seen that obvious differences existed between the two polysaccharides. The precipitation pattern indicated that the TCA-polysaccharide was, probably, a neutral polysaccharide. Cetavlon is an agent more or less specific in its complexing property with acidic polysaccharides. The absence of a precipitate, in this case, indicates either the absence of acidic groups or that the acidic groups are involved in the linkages. These data, coupled with the absence of electrophoretic mobility, led to the conclusion that this TCA-polysaccharide is a neutral polysaccharide.

Studies on the Extracellular Polysaccharide Employing Infra-Red Spectrophotometry

The fractions of the extracellular polysaccharide obtained by alcohol fractionation were examined in aqueous solutions using infra-red spectrophotometry. The spectra are shown in Figure 11. The peaks of the two spectra coincide very well, indicating that there is little, if any, difference between the two fractions of the extracellular polysaccharide.

Table 13. Differences in the Precipitation Reactions of the Extracellular Polysaccharide and the TCA-Polysaccharide.

	Reagent					
Polysaccharide	$CuSO_4$ 7%	Cetavlon 2%	Ethyl alcohol 99.5%	$Ba(OH)_2$ sat.	NaOH 10%	Lead acetate 20%
Extracellular	+[1]	+	+	+	+	+
TCA	-	-	+	+	-	-

[1]This symbol indicates precipitation.

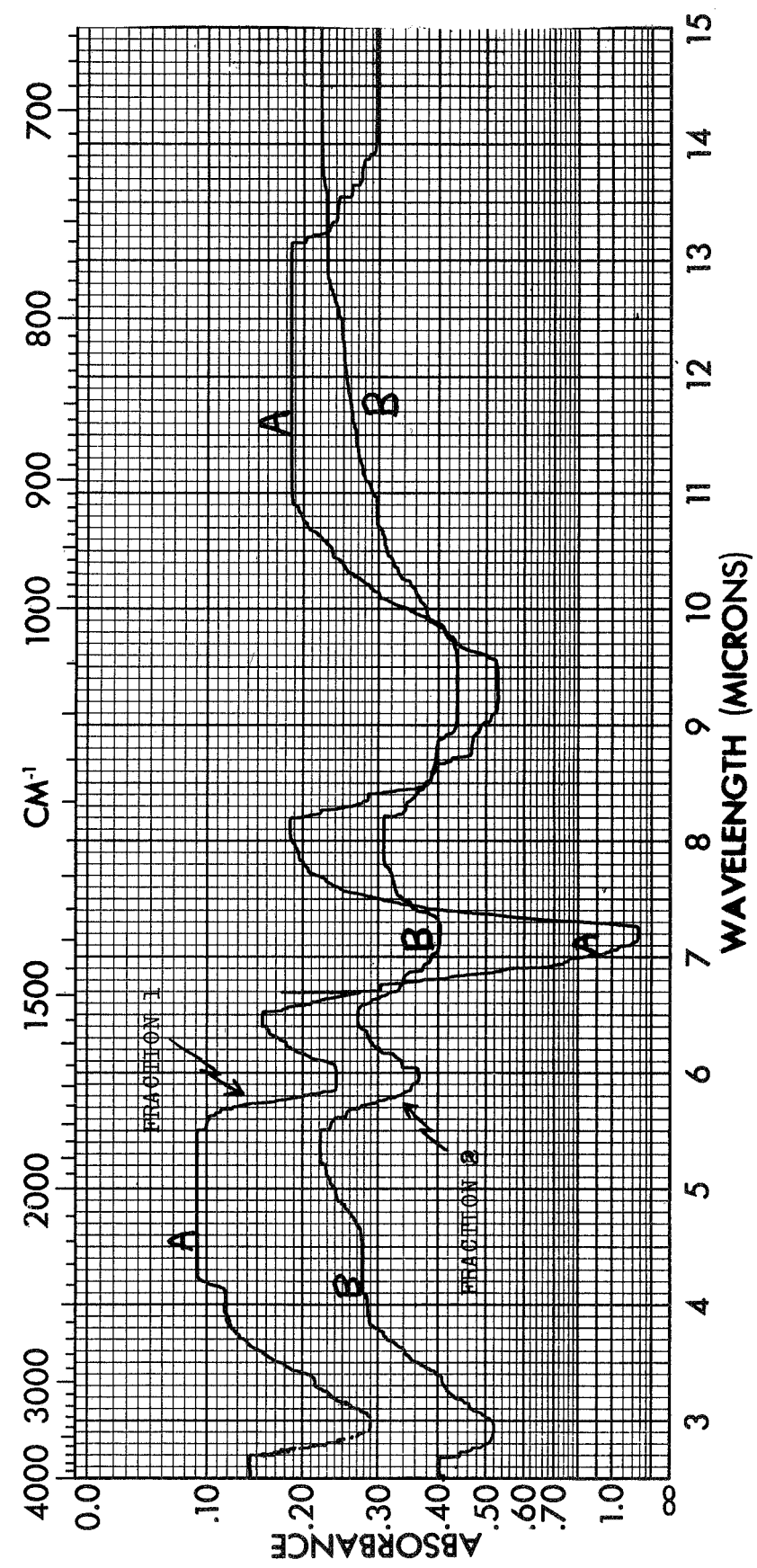


Figure 11. Infra-red Spectra of the Two Fractions of the Extracellular Polysaccharide Obtained by Alcohol Fractionation.

Infra-red spectra were obtained on the unpurified and purified extracellular polysaccharides to determine whether or not any gross differences existed between the two states of the polysaccharides (Figure 12). There was little difference between the two spectra, suggesting that the polymer is in a relatively pure state as it is extracted. The main contaminants appeared to be mineral salts in the medium and these were removed during the purification process.

Studies to Determine the Viscosity of the Polysaccharide Solutions

Aqueous solutions of known concentrations of both the extracellular polysaccharide and the TCA-polysaccharide were subjected to viscosimetric measurements. The time required for the solution to flow and the specific gravity at a constant temperature were determined. The specific gravity values were converted to density values with the aid of tables (ASTM-IP, 1952). The viscosity was calculated in centipoises using the following equation

$$\eta = A\rho t - B(\rho/t)$$ (Daniels, et al., 1962)

where η = viscosity
A = constant for viscosimeter
ρ = density
t = time
B = constant (calculated by $B = \frac{V}{8\pi l}$; where V=volume and l=length).

The results of this study are found in Table 14. It may be seen that the viscosity decreased as the concentration

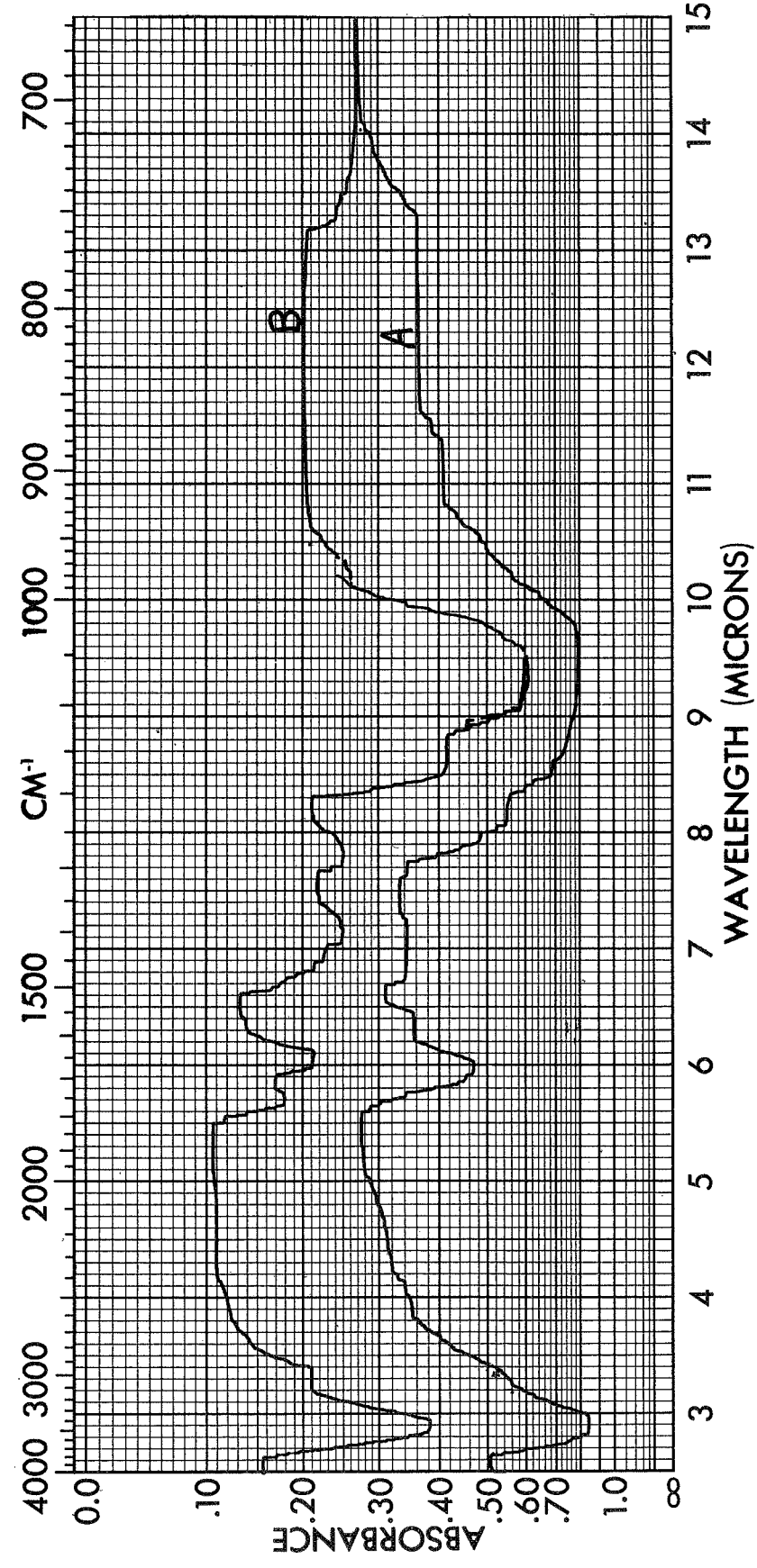


Figure 12. Infra-red Spectra of the Unpurified (A) and Purified (B) Extracellular Polysaccharide.

Table 14. Viscosimetric Determinations on the Extracellular Polysaccharide and the TCA-Polysaccharide.

Polysaccharide	Concentration[1] µg/ml	Viscosity[2] cp[3]
Extracellular	267.0	2.6668
	127.0	1.6308
	31.0	1.0271
TCA	1730.0	1.0377
	173.0	0.8757
	17.0	0.8604
Water		0.9358

[1]The concentration is an average of two determinations. The values were obtained with the phenol-sulfuric acid method.

[2]The time employed in the calculations was the average of five determinations.

[3]cp = centipoises

decreased. The extracellular polysaccharide had a greater density at lower concentrations than did the TCA-polysaccharide. This difference in viscosity again indicates that the polysaccharides are of a different chemical and physical nature.

Study to Determine the Film Characteristics of the Extracellular Polysaccharide

In order to determine the type of film formed by an aqueous solution of the extracellular polysaccharide, a concentrated solution was spread on a glass plate and allowed to dry at room temperature. According to Whistler and Smart (1953) polysaccharides which contain numerous long branches will form only weak and very brittle films which are so fragile that they can not be removed from the plate. On the other hand, the polysaccharides which are linear or have branches of approximately one sugar unit in length form strong, pliable films which can be easily removed. The film formed by the aqueous solution of the extracellular polysaccharide was extremely brittle and impossible to remove from the glass. This information suggested that the polysaccharide contained some long branches.

Study to Determine the Optical Activity of the Extracellular Polysaccharide

Aqueous solutions of both the extracellular (acidic) polysaccharide and the sodium salt of the polysaccharide

were prepared. The solution of the extracellular polysaccharide contained less than 5 mg./ml. and did not completely dissolve. Though the solution was filtered before doing the optical measurement, it was too turbid to obtain an accurate reading. However, all indications were that the substance was optically inactive. The solution of the sodium salt was prepared at a concentration of about 11 mg./ml. This solution was filtered and the optical activity was found to be zero. This indicated that the extracellular polysaccharide was most probably optically inactive.

Serial Hydrolysis of the Extracellular Polysaccharide

A series of tubes containing the polysaccharide and H_2SO_4 was subjected to hydrolysis for different lengths of time to determine the rate of hydrolysis and the monomers which were released. The total carbohydrate content was determined with the phenol-sulfuric acid method and the free sugars were determined using Somogyi's reagent. These results are shown in Table 15. A brief observation of these data reveals that this is not exactly what one might expect from serial hydrolysis. The percent hydrolysis does not increase in proportion to time, but appears to be entirely random and independent of time. This indicates that the polymer is not linear, but that it contains numerous branches.

The hydrolyzates were also subjected to chromatographic analysis to determine the monomers which were released at

Table 15. Serial Hydrolysis of the Extracellular Polysaccharide.

Time of Hydrolysis minutes	Total CH_2O mg	FRS[1] mg	Hydrolysis[2] %
1	10.8	0.16	1.46
3	8.8	1.68	19.10
5	6.8	0.56	8.17
10	9.4	1.80	19.20
20	6.8	1.80	26.50
30	7.4	0.93	12.60
45	10.8	1.86	17.20
60	8.8	0.63	7.16
90	9.2	1.83	19.90
120	8.2	0.85	10.37

[1]FRS = free reducing sugars

[2]The percent hydrolysis was calculated by $\frac{\text{FRS}}{\text{Total } CH_2O} \times 100$.

the various times. This analysis was conducted, employing one dimensional paper chromatography, utilizing the butanol-pyridine-water solvent. The results, here also, were not what would be expected from serial hydrolysis. The separation was not as good as it would have been with two dimensional chromatography; however, all components appeared to be present in each hydrolyzate. The only apparent differences among the various hydrolyzates were the quantities of the monomers which increased as the time of hydrolysis was lengthened. This also appeared to indicate that the polysaccharide is composed of numerous branches which contain all of the monomers in a position subject to removal by a brief exposure to hydrolytic conditions.

SUMMARY

The extracellular polysaccharide produced by the blue-green alga *Anabaena flos-aquae* A-37 has been studied, utilizing a variety of methods, in an attempt to characterize and elucidate the nature of the polymer.

Polysaccharide production increased with the population of the culture and occurred throughout the cultivation period. The ratio of polysaccharide produced to cellular dry weight varied considerably during this time. The ratio was the largest at the beginning of the sampling period which was about the time the culture entered the exponential phase of growth. The ratio dropped to a minimum on about the sixth day after which it began to increase.

It was found that various nitrogen sources had a definite effect on the production of the extracellular polysaccharide. The best yields were obtained when either $Mg(NO_3)_2$, KNO_3 or $NaNO_3$ was employed as the nitrogen source. The least amount of polysaccharide was produced when the alga was fixing nitrogen, probably because of the energy required for the assimilation of this nitrogen source. The pH of the growing cultures probably influenced the quantity produced also.

Solubility trials indicated that the best, and practically only, solvent was water. Attempts to prepare a

more soluble derivative were unsuccessful. Several attempts to partition the extracellular polysaccharide employing the techniques of countercurrent and partition between aqueous polymer phases were also unsuccessful.

Chromatographic analysis of the hydrolyzates revealed that the extracellular polysaccharide was composed of nine, or possibly ten, components. These were identified as uronic acids, glucose, galactose, mannose, arabinose, xylose, fucose, ribose and rhamnose. Glucuronic acid had been previously reported by Moore and Tischer (1965) to be a component and the possibility of galacturonic acid was not excluded since the alga has been shown to possess the enzyme UDP-D-Galacturonic Acid-4-epimerase. The identity of all components except the uronic acids was confirmed.

These results were contradictory to the results previously reported by Moore and Tischer (1965) which listed the components as glucuronic acid, glucose, xylose and ribose. Several attempts were made to resolve this discrepancy; however, no satisfactory explanation was reached since it was not possible to duplicate precisely the conditions under which the previous work was done.

The results of studies to fractionate the extracellular polysaccharide indicated that the polysaccharide was composed of acidic polysaccharides. Investigation of the various fractions revealed that all fractions contained the

same monomers. This led to the conclusion that the polymer was composed of, possibly, several polysaccharides of like composition, differing perhaps in molecular weight. It was not possible to demonstrate the presence of neutral polysaccharides.

It was demonstrated that aqueous solutions of various inorganic and organic compounds had a very definite effect on aqueous solutions of the extracellular polysaccharide. Some of these compounds effected a precipitation of the polysaccharide by the formation of a salt while others effected a clarification of the solution. Compounds such as NaOH, $Ca(OH)_2$, lead acetate, $CuSO_4$, $MgSO_4$, among others, were found to precipitate the polysaccharide. Most of these precipitates were insoluble in water. Compounds such as Mg citrate, H_3PO_4, EDTA and $(NH_4)_2SO_4$ were found to bring about a clarification of the solution.

Disc electrophoretic techniques were employed to determine if the polysaccharide could be fractionated electrophoretically. Only one band was detected after electrophoresis. The fractions obtained by alcohol fractionation were subjected to electrophoresis and it was found that no electrophoretic difference existed between the fractions. It was found that a slight difference existed in the mobility of the extracellular polysaccharide and the sodium salt; however, when these were combined the two

moved as a single substance. The results of these studies indicated that the polysaccharide was electrophoretically homogeneous.

Washed cells of the alga were extracted with trichloroacetic acid to yield another polysaccharide. The polysaccharide had no electrophoretic mobility. It also gave different reactions with several of the reagents previously tested on the extracellular polysaccharide. The conclusion drawn from these results was that this polysaccharide was a neutral polysaccharide which differed considerably from the extracellular polysaccharide.

Infra-red spectrophotometric studies indicated that little, if any, difference existed between the fractions obtained by alcohol fractionation. There was also found to be little difference in the spectra of the unpurified and purified samples of the extracellular polysaccharide, indicating that the substance is in a relatively pure state upon extraction.

Viscosimetric studies revealed that the viscosity is proportional to the concentration of the polysaccharide. The viscosity of the extracellular polysaccharide and the TCA-polysaccharide was compared. It was found that the extracellular polysaccharide had a greater viscosity, even at lower concentrations, than the TCA-polysaccharide. This tends to confirm the previous conclusion that a difference

exists between the two substances.

The type of film formed by an aqueous solution of the extracellular polysaccharide was determined in order to estimate the structural characteristics of the polysaccharide. The characteristics of the film indicated that the polysaccharide contained branches, possibly long and numerous.

Optical activity measurements on the extracellular polysaccharide and the sodium salt indicated that the substances were optically inactive.

Studies employing serial hydrolysis of the extracellular polysaccharide revealed some interesting and rather unexpected results. It was found that the hydrolytic pattern was random and independent of time. Chromatographic analyses revealed that all components were released at different time intervals. The only apparent difference among the various hydrolyzates was in the concentration of the monomers. These results indicated that the polymer was composed of numerous branches with all components in a position tp be removed after only a brief exposure to hydrolytic conditions.

In the final analysis of all results, it was concluded that the extracellular polysaccharide produced by the blue-green alga Anabaena flos-aquae A-37 is an acidic polysaccharide which is chemically and electrophoretically

homogeneous and that it structurally contains numerous branches. The polysaccharide constituents were revealed to be: uronic acids, glucose, galactose, mannose, arabinose, xylose, fucose, ribose and rhamnose.

Table 16. Summary Table of the Characteristics of the Extracellular Polysaccharide Produced by *A. flos-aquae* A-37.

Characteristic	Result
Solubility	Water soluble
Quantity	Influenced by the nitrogen source and pH
Nature of polymer	Composed of acidic polysaccharides
Constituents	Uronic acids Glucose Galactose Mannose Arabinose Xylose Fucose Ribose Rhamnose
Homogeneity	Chemically and electrophoretically homogeneous
Precipitated by	Cetavlon (Cetyltrimethylammonium bromide) NaOH $MgSO_4$ $CuSO_4$ Lead acetate Ethyl alcohol $Ca(OH)_2$
Structure	Complex heteropolysaccharide with numerous branches
Viscosity	Proportional to the concentration
Optical activity	Inactive

ABSTRACT

Edith Boyanton Davis, Doctor of Philosophy, 1970

Major: Microbiology

Title of Dissertation: "Characterization Studies on an Extracellular Heteropolysaccharide Produced by Anabaena flos-aquae A-37"

Directed by: Dr. Robert G. Tischer

Pages in Dissertation: 101

Words in Abstract: 576

ABSTRACT

These studies were undertaken to elucidate the characteristics of an extracellular polysaccharide produced by a bacteria-free culture of the blue-green alga Anabaena flos-aquae A-37.

Production of extracellular polysaccharide occurred throughout the cultivation period. The ratio of mg polysaccharide produced to mg cellular dry weight varied during this time.

Various nitrogen sources exerted a definite effect on the quantity of polysaccharide produced. The best yields were obtained when either $Mg(NO_3)_2$, KNO_3 or $NaNO_3$ was utilized. The least amount of polysaccharide was produced when the alga was fixing nitrogen. The quantity of polysaccharide produced is influenced by the nitrogen source,

pH and the extent of growth.

Solubility tests indicated that the best solvent for the polysaccharide was water.

Chromatographic analyses of polysaccharide hydrolyzates revealed that the constituents were uronic acids, glucose, galactose, mannose, arabinose, xylose, fucose, ribose and rhamnose. Earlier reports listed the components of this polysaccharide as glucose, xylose, ribose and glucuronic acid. Several attempts were made to resolve this discrepancy; however, no satisfactory explanation was reached.

Fractionation studies revealed that the polymer was composed of acidic polysaccharides. All of the fractions, examined chromatographically, contained the same monomers. This led to the conclusion that the polymer was composed of, possibly, several polysaccharides of like composition, differing perhaps in molecular weight. Neutral polysaccharides were not detected.

It was demonstrated that aqueous solutions of various inorganic and organic compounds had a very definite effect on aqueous solutions of the polysaccharide. Compounds such as NaOH, $Ca(OH)_2$, $CuSO_4$ and $MgSO_4$ effected the formation of a precipitate. Other compounds such as Mg citrate, EDTA, H_3PO_4 and $(NH_4)_2SO_4$ effected a clarification of the solution.

Disc electrophoresis studies indicated that the extracellular polysaccharide was electrophoretically homogeneous.

All of the fractions exhibited the same electrophoretic mobility. There was a slight difference between the mobility of the extracellular (acidic) polysaccharide and the sodium salt; however, when combined the two moved as a single substance.

The polysaccharide extracted from washed cells with trichloroacetic acid was found to differ from the extracellular polysaccharide. This TCA-polysaccharide did not exhibit any electrophoretic mobility. A different reaction pattern with the reagents tested on the extracellular polysaccharide was obtained. These results indicated the this was a neutral polysaccharide.

Infra-red studies conducted on the fractions of the extracellular polysaccharide indicated that little difference existed between the fractions. It was also found that the spectra of the unpurified and purified extracellular polysaccharides differed very little. This appeared to indicate that the substance was in a relatively pure state upon extraction.

Viscosimetric determinations on the extracellular polysaccharide and the TCA-polysaccharide revealed that the viscosity was proportional to the concentration. The viscosity of the TCA-polysaccharide was found to be less even at greater concentrations.

Film formation was employed to estimate the structural

characteristics of the extracellular polysaccharide. The characteristics of the film formed indicated that the polysaccharides contained numerous branches.

Both the acidic polysaccharide and the sodium salt were found to be optically inactive.

Serial hydrolysis of the extracellular polysaccharide revealed that the hydrolytic pattern was random and independent of time. All monomers were present in each hydrolyzate regardless of the duration of hydrolysis. These results indicated that the polysaccharide was composed of numerous branches with all constituents in a position to be removed after a brief exposure to hydrolytic conditions.

The extracellular polysaccharide produced by Anabaena flos-aquae A-37 was concluded to be an acidic polysaccharide which is chemically and electrophoretically homogeneous and that it structurally contains numerous branches. The constituents were revealed to be: uronic acids, glucose, galactose, mannose, arabinose, xylose, fucose, ribose and rhamnose.

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