Preparation of Purified Polysaccharides from *Rhizobium*

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Abstract

Davis, R. J. (U. S. Department of Agriculture, Beltsville, Md.), and C. E. Clapp. Preparation of purified polysaccharides from *Rhizobium*. Appl. Microbiol. **9**:519–524. 1961.—A method is described for the preparation of purified polysaccharides from strains of *Rhizobium* in quantities large enough so that with an exhaustive purification scheme enough product is recovered for various characterization purposes. When steps in the purification process are eliminated, much larger amounts of crude gum are obtained.

Organisms were grown in liquid medium and the crude gum was precipitated along with the bacterial cells by a quaternary ammonium complexing agent. This precipitate was dissolved in salt solution, reprecipitated with ethanol or ethanol-acetone mixtures several times, followed by pressure filtration with membrane filters.

The same procedure should be applicable to commercial scale production in large fermentors if a use for the gum could be shown. The method also should be suitable for other organisms with similar growth habits.

The gums produced by the root nodule bacteria, genus *Rhizobium*, of leguminous plants are of interest both from the view of bacteriology and of soil science. Study of these compounds has been hampered by lack of suitable methods for the production of a purified product in amounts large enough for characterization.

Review of the scant work done on these compounds reveals that most workers either have grown the organisms on solid medium, then washed off the growth and purified the product, or have grown them in liquid culture with the large ratio of container volume to culture volume (Bray, Schlüchterer, and Stacey, 1944; Cooper, Daker, and Stacey, 1938; Anderson, 1933). Aside from the inconvenience of growing large quantities in this manner, with solid media the possibility of contamination from agar is significant (Bray et al., 1944; Yaphe, 1957, 1958). Variations to eliminate the problem of agar contamination (Bray et al., 1944) are tedious and do not lend themselves to large-scale production.

Growth in liquid medium followed by isolation and purified by classical precipitation techniques requires either that large and unwieldy volumes are encountered or else the material must be concentrated. This latter course, in addition to being time consuming and tedious however done, also leads to the danger of denaturization of the polysaccharides and disruption of cells unless accomplished by lyophilization. In addition it is found that classical precipitation techniques fail to bring about precipitation of the gum produced by some strains. Earlier workers ignored such strains when they encountered them and picked specifically strains producing copious amounts of gum. This technique is allowable if the sole purpose is to study new types of gum, but untenable if a true cross section of the gums produced by this genus is to be obtained.

The method presented below has been found suitable for the production of gums by growing organisms in volumes up to 20-liter carboys. With the equipment in our laboratory the method could easily be upgraded to growth in a 50-liter carboy and it should be feasible for commercial scale production of these materials if a need should arise.

These materials have been analyzed for purity and composition. Their characterization by various chemical and physical means is the subject of a subsequent paper.

Materials and Methods

Medium. Cultures of *Rhizobium* were grown in liquid medium, a variation of the standard yeast extract-mannitol medium, of the following composition: $MgSO_2 \cdot 7H_2O$ and $CaCl_2$, to give a concentration 0.003 M with respect to Ca and to Mg; tris(hydroxymethyl)aminomethane (tris) buffer (pH 6.8), 0.1 M final concentration; mannitol, 5%; and yeast extract (50 g starch-free active dry yeast steamed with water, made to 1-liter volume and filtered twice through S & S no. 588[1] filter paper), 30 ml per liter of medium. The advantages of a completely synthetic medium were recognized, but the above medium was chosen because good growth of any strain in our stock cultures would occur, whereas optimal growth cannot always be obtained with synthetic media. This yeast extract concentration is less than our standard medium because it has been observed that too much nitrogen in the medium sometimes will reduce gum production.

[1] Schleicher and Schuell Company, Keene, N. H.

The mannitol content is higher for obvious reasons. Since *Rhizobium* cultures will produce gum from almost any carbon source it can be assumed that hexoses are degraded and rebuilt from shorter chain carbon skeletons. Mannitol was used only because it has been extensively used for growing these organisms and all of our stock cultures were being carried upon it.

Growing cultures. Cultures were grown with 3 liters of solution in a 4-liter reagent bottle or 10-liters in a 20-liter carboy. Incubation was at 28 C. Sufficient aeration was obtained in the 4-liter bottles by means of a magnetic stirrer with a 3-in. Teflon-coated stirring bar which was added to the bottle before autoclaving. Forced aeration was necessary with the 20-liter size. Figure 1 shows the apparatus used. Aeration was accomplished by using a small pump as an air source. Tygon tubing conducted air from pump to culture jar and a sintered-glass filter stick dispersed the air into the medium. Two in-line filter holders were placed in the air line between the pump and the culture medium. The first contained a glass fiber filter; the second a membrane type filter. The entire unit from the filter on could be autoclaved as a unit. The air intake line to the pump had an open end type filter holder containing a glass fiber filter. This filter which could be changed without contaminating the system scrubbed the air of most of the debris before it reached the in-line filters, precluding the need to tamper with the sterile part of the system during a run. Air pressure was adjusted daily through a bleeder vent and the scrubber filter was changed when air flow decreased below the desired level. Check runs with enriched media did not become contaminated.

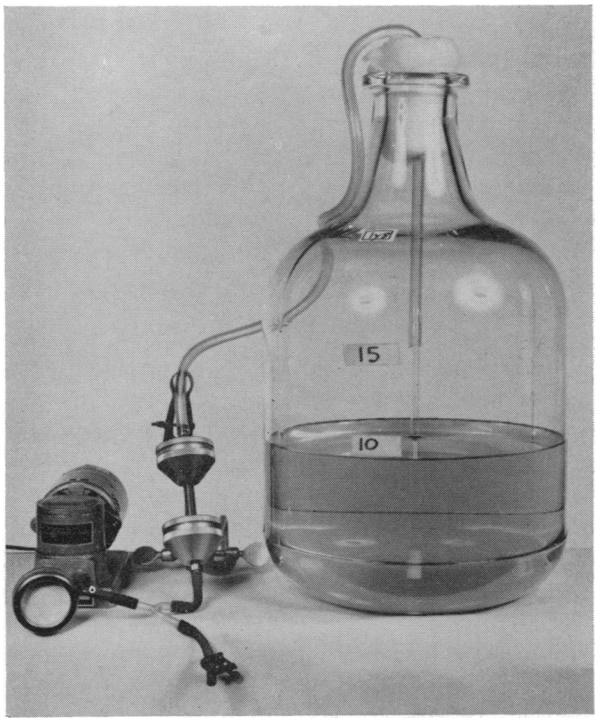

FIG. 1. *Culture apparatus showing pump with filter attached to air intake line to scrub air of most debris (this filter can be changed when clogged without opening sterile system), bleeder valve to adjust pressure, two in-line filters (the first with glass fiber filter, the second with membrane type filter), Tygon tubing to transport air to culture, and fritted glass filter stick to disperse air into the culture. The bottle, tubing and in-line filters can be autoclaved as a unit.*

The entire apparatus was placed in a reach-in type constant temperature chamber with built-in electrical outlets. There was therefore no need to run lines of any type from the outside, and heat generated by the pump or stirring motors was removed effectively.

Preparation of inocula. Inoculation was with 100 ml of 3- to 5-day starter cultures. Bottles were inoculated aseptically in a transfer chamber before being placed in the incubation chamber.

Harvesting. The general outline of the harvesting and purification steps is shown in Fig. 2. Harvesting of the gums was accomplished by addition of the quaternary ammonium compound, hexadecyltrimethylammonium bromide, to the culture. Use of long-chain quaternary ammonium compounds in the isolation and purification of acidic polysaccharides has recently been reviewed (Scott, 1960) so theoretical details will not be considered here. A precipitate containing both cells and gum appeared instantly with most cultures and usually would settle out rapidly, although some of the more fluffy ones required several hours. Two grams of quaternary per liter of solution were sufficient to cause complete precipitation in most cases. However, each culture was checked for complete precipitation at this point by addition of another gram of complexing agent before proceeding further. In addition to checking for completeness of precipitation, the excess quaternary should also dissolve any precipitated protein contaminant (Scott, 1955). The gross appearance of the precipitates varied from one strain of organisms to another and a few produced such a bouyant floc that it rose to the top rather than settling to the bottom of the bottle. A typical precipitation is shown in Fig. 3.

The large volume of liquid, after settling of the precipitate, was decanted or siphoned off and saved. The remainder of the supernatant was removed by centrifugation. After removal of the above precipitate (type I), a further precipitate (type II) was produced by adding a few KOH pellets. With some strains there was no perceptible precipitation until KOH was added (no type I); other times a negligible amount of further precipitation appeared with an addition of KOH (no type II). Reports in the literature (Palmstierna, Scott, and Gardell, 1957) indicate appreciable amounts of KOH are necessary for complete precipita-

tion. While it is recognized that appreciable is a relative term, the amounts required would not be so considered by us.

Preparation of purified polysaccharide. The precipitates were washed several times to remove excess complexing agents by suspending in water and centrifuging. The materials were freeze-dried prior to storage. Purification was accomplished by extracting with 10% NaCl in the case of the type I complexes and with 2 M acetic acid in the case of the type II complexes. Early attempts to keep the NaCl concentration to a minimum to avoid dissolving other components as reported in the literature (Jones, 1953; Scott, 1956) were abandoned for the following reasons. There were not appreciable amounts of other hexadecyltrimethylammonium bromide-precipitable materials in the cultures. In addition, many preparations could not be extracted completely by 2% NaCl. It was simpler, therefore, to extract all samples with 10% NaCl than to extract each one in a minimal concentration. The crude polysaccharide was precipitated by pouring the NaCl or acetic acid extract into two volumes of 1:1 ethanol-acetone. All preparations would precipitate polysaccharides from acetone or ethanol-acetone, whereas ethanol alone did not produce complete precipitation.

Many preparations would produce precipitates in the cold that would not precipitate at room temperature, therefore, samples were routinely put in the freezer compartment of the refrigerator before decanting or centrifuging to remove the supernatant. Polysaccharides, precipitated at room temperature or warmed up by centrifugation in a nonrefrigerated centrifuge, would often produce further precipitation from the supernatant when allowed to stand in the cold for a few hours. The proper concentration of ethanol:acetone:water for maximal precipitation was almost an individual case with each preparation so no firm rule can be given as to the best mixture to use.

The first precipitation removed enough complexing agent so that the material was water soluble. After centrifugation, the polysaccharides were reprecipitated from ethanol-acetone mixtures. Several precipitations and centrifugations were necessary to remove all debris. During reprecipitation it often was found that only a gel or suspension would result. Concentration by rotary flash evaporation usually served to produce an effective precipitation. With low yielding preparations, it was necessary to concentrate the supernatant after centrifugation to obtain maximal polysaccharide recovery. In some troublesome cases, it was found that acidifying the suspension with acetic acid would flocculate and precipitate the polysaccharide.

At this point most of the residual soluble contamina-

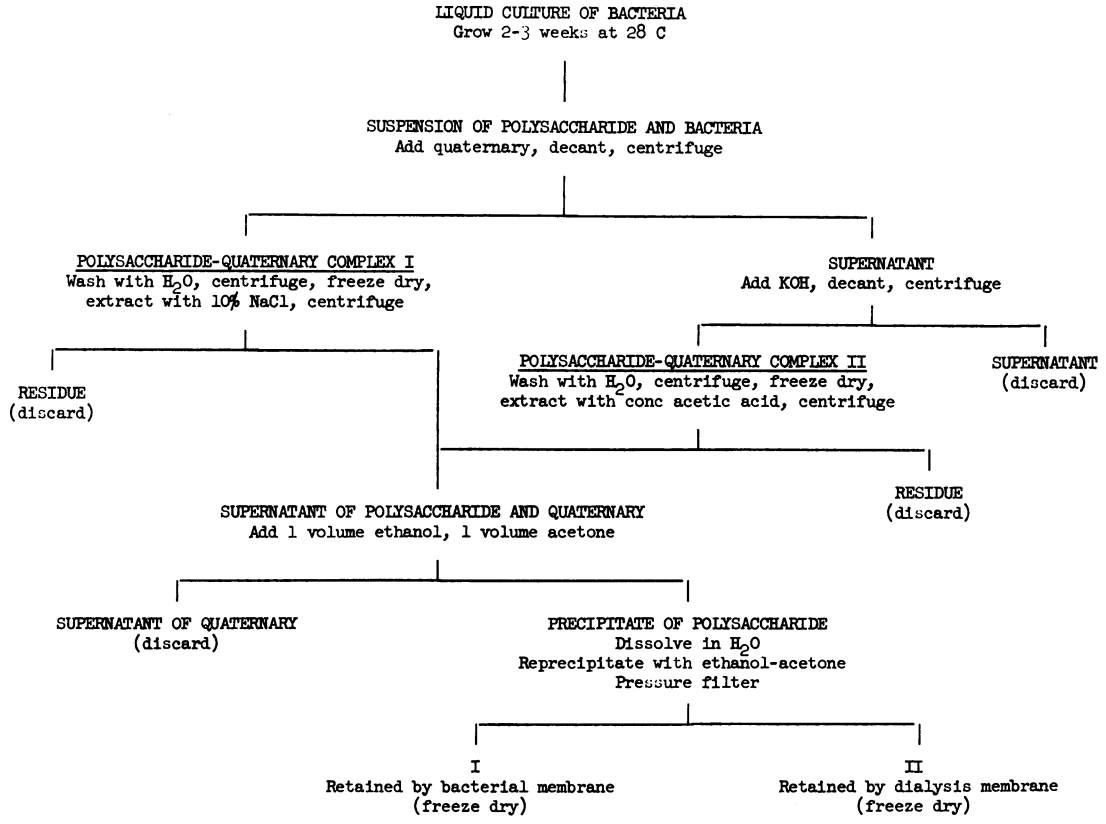

FIG. 2. *Outline of procedure for preparation of purified rhizobial polysaccharides*

tion was removed by pressure filtration with membrane filters, using Lucite cells and applying 3 to 5 atm of compressed nitrogen. The pressure apparatus is shown in Fig. 4. Filtration through S & S coarse, graded-porosity membrane filters with type I preparations removed salts, monosaccharides, and low molecular weight components. The polysaccharide precipitated as a mat on the membrane. The type II precipitates were divided into two components by the coarse filter, one which passed through and one which did not. The material which passed through the coarse filter was then filtered through a dialysis membrane to remove the low molecular weight components. The material which did not pass through the coarse filter had to be further purified by dissolution and centrifugation to remove any residual cells.

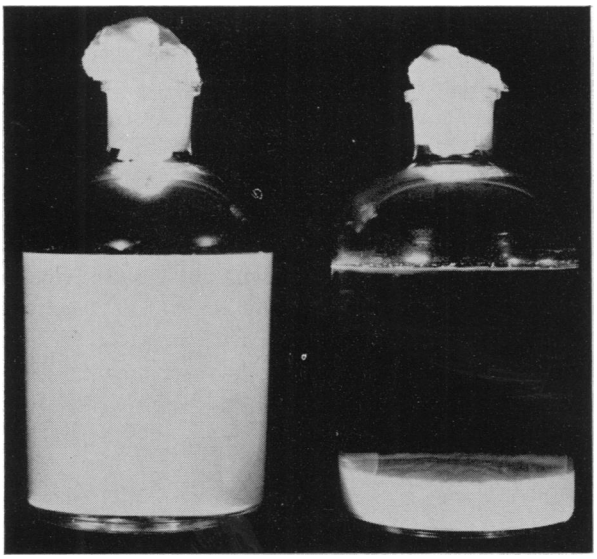

FIG. 3. *Typical precipitation of a bacterial culture with quaternary ammonium compound. Bottle on left: unprecipitated culture; bottle on right: culture with quaternary added.*

RESULTS

All strains of *Rhizobium* checked showed the same reaction toward all quaternary compounds tested. Use of hexadecyltrimethylammonium bromide, therefore, was only in the interest of standardization. This compound was chosen arbitrarily over the others for the following reasons: it has been more widely used than other quaternary compounds, is readily available commercially, and is supplied as a purified product which is easily handled. Complexing agents, checked and found identical in reaction of hexadecyltrimethylammonium bromide against representative strains of each type of precipitation reaction noted, included ethylhexadecyldimethlyammonium bromide, hexadecyldimethylbenzylammonium chloride, hexadecylpyridinium bromide, dodecylpyridinium bromide, and dodecyltrimethylammonium chloride. Many quaternaries listed in commercial catalogues were found to be unavailable when ordered.

Reaction of strains of *Rhizobium* to various precipitating agents are shown in Table 1. These observations were taken at 1 min. On longer standing, all strains so far checked were precipitated with addition of KOH to quaternary suspension except the red strain of Norris (1958). This strain produced very scant growth which settled out on standing, leaving a clear solution. It is thought that this strain produced no extracellular polysaccharide to be precipitated. One strain produced a water-insoluble fraction which could be dissolved in cuprous ammonium solution. *Rhizobium* strains, when

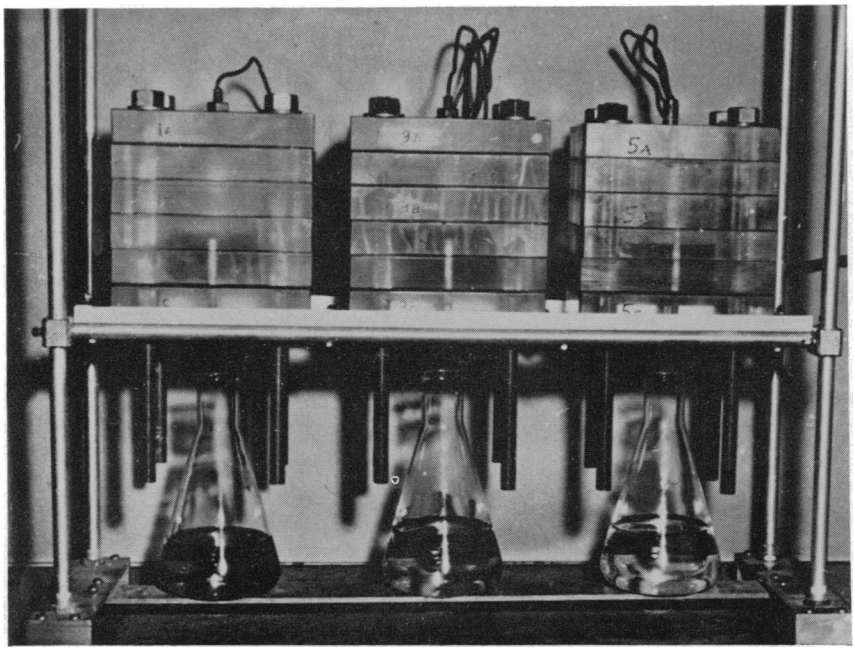

FIG. 4. *Pressure filtration apparatus. The membrane filter was placed in a depression in the bottom of the cell on top of a plastic grid. The Lucite cell was bolted together and pressure was applied to the top from a tank of compressed nitrogen.*

grown in liquid cultural medium without shaking, produced several types of growth patterns, i.e., cells stay throughout the medium, a large percentage of the cells settle to the bottom, there is slight pellicle formation, a tendency to form clumps which settle out, and combinations of these. The final pH of the growth medium, if not adjusted during growth, may remain unchanged, be slightly acid (pH 6 to 7), moderately acid (pH 5 to 6), or slightly basic (pH 7 to 8). There was no relationship between these growth characteristics and the reaction of a given strain to various precipitating agents. Adjusting the pH to the original value did not change the pattern of precipitation.

TABLE 1. *Characterization of strains of Rhizobium with respect to behavior toward various precipitating agents**

Precipitant†	*Rhizobium* cross-inoculation group‡						
	Alfalfa	Soybean	Lupine	Clover	Pea	Cowpea	Others
1+	22	0	0	3	3	1	5
1−	2	8	0	8	6	3	2
2+	19	0	0	3	1	1	4
2−	3	0	0	0	2	0	1
3+	1	2	2	6	2	2	0
3−	1	6	0	2	1	1	1
4+	0	0	0	1	6	0	1
4−	1	8	0	1	0	6	0
5+	24	0	0	3	3	1	6
5−	0	8	2	8	0	3	1
6+	24	0	0	4	3	1	6
6−	0	8	2	7	0	3	1

* Bacteria were grown in liquid media for 1 week and precipitated in situ. Observations were made at 1 min.

† 1 = Hexadecyltrimethylammonium bromide, 5%. Negative ones also negative with 20% solution; 2 = further precipitation on adding KOH to supernatant from precipitate in 1 above; 3 = precipitate with hexadecyltrimethylammonium bromide plus KOH but none with hexadecyltrimethylammonium bromide alone; 4 = precipitate with hexadecyltrimethylammonium bromide plus sodium tetraborate if no precipitate with any of first three; 5 = 95% ethanol; 6 = acetone.

‡ Figures indicate number of strains giving reaction indicated.

TABLE 2. *Rhizobium strains from which purified polysaccharides have been prepared with yield of purified polysaccharides*

Culture no.	Cross-inoculation group	Source and date	Yield of polysaccharide
			mg/liter
3C1a2	Crotalaria	Maryland, 1932	77
3C1k3	Crotalaria	Florida, 1943	480
3C2d3a	Lupine	Georgia, 1946	138
3D0a8	Alfalfa	Maryland, 1933	140
3D0a13	Alfalfa	New Jersey, 1939	200
3D0a31	Alfalfa	Turkey, 1952	265
3D1m32	Clover	Georgia, 1953	49
3D1q24	Clover	Maryland, 1954	24
3D1y8	Clover	Turkey, 1952	742
SU298/533	Clover	Australia, 1950	88
3E0a3	Lotus	Ohio, 1941	214
3E0l2	Lotus	Maryland, 1946	360
3F1e4	Indigofera	Florida, 1954	21
3H0q13	Pea	Michigan, 1938	397
3H0q47a	Pea	Iowa, 1948	78
3I1b66	Soybean	North Carolina, 1948	52
3I1b118	Soybean	Maryland, 1936	96
3I6l3	Bean	Virginia, 1919	11

Table 2 shows the cultures from which polysaccharides have been isolated and carried through the complete purification scheme together with the cross-inoculation group represented, source, and date of original isolation and yield of purified polysaccharide.

Discussion

The procedures described in this paper give a relatively simple method for the production of amounts of extracellular polysaccharide sufficient for characterization procedures. These organisms are not extremely copious gum producers and the gum adheres tenaciously to the cells. The nature of the gum itself causes large amounts of material to be lost in the purification process. This loss is often proportionally more with the more sparse gum producers. The final yield of polysaccharide is low in many cases but represents a highly purified product suitable for use in characterization procedures. For uses where the product of the first or second precipitation would suffice, the yield would be many times greater. This procedure should be applicable to other organisms with similar growth habits.

The findings in the precipitation scheme are somewhat anomalous. It was originally postulated that gums containing charged moieties would precipitate with quaternaries and noncharged ones with quaternary-borate complexes. However, all strains precipitated with quaternary or quaternary plus KOH, whereas most strains that would not precipitate with quaternary alone would not precipitate with quaternary plus borate.

The use of a complexing agent, even with those strains which produced gum precipitable with ethanol, has the extreme advantage of eliminating the need for either working with large volumes or concentrating the cultures before precipitation since the complexing agent will remove very completely all material from a large volume of culture.

Theoretically, all polysaccharide material should pass through the coarse membrane filter. If this were the case, purification could be accomplished by a two-step filtration process. Filtration of the solution of partially purified material through the coarse filter would eliminate any residual cells at this stage and eliminate the need for further reprecipitations and centrifugations to remove the last traces of these. Filtration of the material passing through the coarse

filter through dialysis membrane would eliminate the low molecular weight components and retain the polysaccharide inside the cell. Bringing the material inside the cell to volume several times would leave essentially pure polysaccharide inside. As it turned out only part of the type II precipitates would pass through the coarse membrane. All type I precipitates were retained almost quantitatively by the membrane. After only a very small fraction of the solution had passed through the filter, a mat would form over the membrane. No feasible way was found to eliminate formation of this mat. It would appear that a lattice type structure was formed which fit very closely to the filter membrane, probably held securely by electrostatic charges, which reduced the effective pore size of the membrane.

LITERATURE CITED

ANDERSON, D. A. 1933. The production of gum by certain species of *Rhizobium*. Iowa Agri. Expt. Sta. Research Bull. No. 158, 27–56.

BRAY, H. G., E. SCHLÜCHTERER, AND M. STACEY. 1944. Enzyme formation and polysaccharide synthesis by bacteria. 2. Polysaccharide formation by *Rhizobium radicicolum* strains. Biochem. J. **38**:154–156.

COOPER, E. A., W. D. DAKER, AND M. STACEY. 1938. Enzyme formation and polysaccharide synthesis by bacteria. 3. Polysaccharide produced by "nitrogen-fixing" organisms. Biochem. J. **32**:1752–1758.

JONES, A. S. 1953. The isolation of bacterial nucleic acids using cetyltrimethylammonium bromide (Cetavlon). Biochim. Biophys. Acta **10**:607–612.

NORRIS, D. O. 1958. A red strain of Rhizobium from *Lotononis bainesii* Baker. Australian J. Agr. Research **9**:629–632.

PALMSTIERNA, H., J. E. SCOTT, AND S. GARDELL. 1957. The precipitation of neutral polysaccharides by cationic detergents. Acta Chem. Scand. **11**:1792–1793.

SCOTT, J. E. 1955. The reaction of long-chain quaternary ammonium salts with acidic polysaccharides. Chem. & Ind., p. 168–169.

SCOTT, J. E. 1956. The preparation and fractionation of acidic polysaccharides using long-chain quaternary ammonium compounds. Biochem. J. **62**:31 p.

SCOTT, J. E. 1960. Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues. Methods of Biochem. Anal. **8**:145–195.

YAPHE, W. 1957. The use of agarase from *Pseudomonas atlantica* in the identification of agar in marine algae. Can. J. Microbiol. **3**:987–993.

YAPHE, W. 1958. Standards for bacteriological agar. Bacteriol. Proc., p. 26.

In Vitro Activity of Actinospectacin in Human Whole Blood

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ABSTRACT

SOKOLSKI, W. T. (The Upjohn Company, Kalamazoo, Mich.), C. G. CHIDESTER, AND L. K. SCHADEWALD. In vitro activity of actinospectacin in human whole blood. Appl. Microbiol. **9**:524–528. 1961.—A method for testing antibacterial substances in whole blood is described. The test agent for the method was actinospectacin which reportedly has good in vivo activity, approximately in the range with chloramphenicol, but relatively poor in vitro activity in the common media. In human whole blood, however, the in vitro activity compares favorably with chloramphenicol thus indicating that whole blood may predict in vivo activity better than the usual bacteriological media.

Some biological properties of actinospectacin, a new basic antibiotic, were recently reported by Mason, Smith, and Dietz (1961) and by Lewis and Clapp (1961). Although actinospectacin is active in vitro and in vivo against a variety of gram-positive and gram-negative organisms, it elicited a greater response in vivo than might be expected from its in vitro activities in brain heart infusion broth (Lewis and Clapp, 1961).

An in vitro test system for actinospectacin was desired for the purpose of predicting in vivo responses against pathogenic bacteria. For this, a medium was sought which would simulate in vivo environment. Studies with the use of blood serum as a complete antibiotic testing medium have been reported in the past (Sokolski, Vavra, and Hanka, 1960; Wolfe and McGuire, 1961). A blood medium which would include the cells may be an improvement since it is a step closer to the in vivo environment than serum.

This paper describes a method for testing antibacterial substances in human whole blood and the results of comparative tests with two antibiotics, actinospectacin and chloramphenicol. Actinospectacin with poor in vitro and good in vivo activity is compared to chloramphenicol with good in vitro and in vivo activity (Smith et al., 1948). If whole blood is an