CCXXVII. ENZYME FORMATION AND POLYSACCHARIDE SYNTHESIS BY BACTERIA

III. POLYSACCHARIDES PRODUCED BY "NITROGEN-FIXING" ORGANISMS

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In a previous paper [Cooper & Preston, 1937] attention has been drawn to the question of polysaccharide formation by the nitrogen-fixing organisms, *Rhizobium radicicolum*, the root-nodule bacterium associated with the Leguminosae, and the soil organism, *Azotobacter chroococcum*. In the present paper we have confirmed the previous observations and have obtained preliminary data on the purification and properties of these interesting polysaccharides. In themselves these products are of considerable importance since they probably constitute a certain proportion of the so-called "humic substances" in soil. From our point of view they are typical examples of nitrogen-free bacterial mucilages. As already indicated, a study of the chemical and physical properties of the *Rhizobium* polysaccharides and the recognition of glucose and uronic acids among their cleavage products places them in the same class of compound as the specific polysaccharides of types II and III *Pneumococcus*. We have now shown that the *Azotobacter* polysaccharide also belongs to the same class. As a result of further study of the metabolism of these organisms, the polysaccharides are now reasonably accessible and in such quantities as to enable their chemical investigations along classical lines to be undertaken. Later it is hoped to apply the information gained to research on important questions of immunological specificity.

In this study, careful attention has been given also to the urgent problem of the possible contamination of specific polysaccharides by extraneous carbohydrate material from the media on which the organisms are cultivated. It is generally known that organisms grown on an agar medium are liable to extensive contamination with agar-polysaccharides. This danger has been particularly emphasized by Morgan [1936] in the case of the specific polysaccharide of *B. dysenteriae*. In their studies on a mucoid polysaccharide from *Streptococcus*, Heidelberger *et al.* [1937] were careful to show that no polysaccharides from the sterile broth medium were precipitated under the conditions used for isolating the bacterial polysaccharide.

In order to minimize the danger of contamination due to mechanical dislodging of agar particles we raised the agar concentration in the medium to 1·7%. In Roux bottles this gave a comparatively hard surface at 37°, and after incubation for several days the washings from these bottles were shown to contain a negligible amount of agar. Agar is constituted mainly of galactose residues so that we were particularly fortunate in being able to show that the poly-

saccharides from the nitrogen-fixing organisms were free from galactose. Of greater importance, however, was the observation that the organisms formed the polysaccharide characteristic of each on either agar or gelatin media; so it is considered that our purified products are free from extraneous carbohydrates.

Two other nitrogen-fixing organisms, *B. urea* (Beijerinck) and *B. trauffauttii*, were investigated in regard to polysaccharide formation. They grew vigorously on an agar medium but when washed off and precipitated no polysaccharides could be isolated.

Rh. radicicolum *polysaccharide*

The organism was an active culture of the clover strain kindly supplied by Dr H. Nichol of Rothamsted Experimental Station.

Extensive investigations on the growth of the organism in liquid media did not lead to a satisfactory method of polysaccharide production. The viscous material obtained was very difficult to purify and the yields were poor. On solid media, however, growth was vigorous and resulted in prolific polysaccharide formation which, moreover, was continuous over a long period. The striking advantage in the production of this type of polysaccharide on solid media may have an important application in large scale preparation of those pathogenic organisms whose antigenic properties may depend on the elaboration of complex polysaccharides.

After numerous investigations of sources of available nitrogen and carbon and of possible growth stimulators, a reliable agar medium for producing the polysaccharide was established. On a gelatin medium the same polysaccharide was produced but the growth was slower and the yield inferior.

Crude *Rh. radicicolum* polysaccharide was grossly contaminated with water-insoluble material, tenaciously held in colloidal suspension. Much of this impurity consisted of bacterial protein the greater part of which was removed by repeated filtration of a dilute aqueous solution through a pad of kieselguhr. Final traces were eliminated by exhaustive alcoholic fractionation. A typical sample of the polysaccharide was obtained as a white fibrous mass $[\alpha]_D - 17^\circ$ in water; ash, 2%; uronic anhydride, 18%. Its hydrolysis with N H_2SO_4 was complete in about 8 hr. at 100°. From the hydrolysate ($[\alpha]_D + 18^\circ$) crystalline *d*-glucose (67% yield) and the barium salt of a uronic acid (23% yield) were isolated. The latter had $[\alpha]_D + 11^\circ$, Ba 16·0%, and was probably the salt of an aldobionic acid. Further investigations of this acid and the polysaccharide will be reported later.

The Azotobacter *polysaccharide*

The organism used in this study was a culture of *Azotobacter chroococcum* freshly isolated from soil and which gave a vigorous production of polysaccharide on gelatin or agar slopes. Initial investigation of its metabolism was directed towards the finding of a suitable liquid medium in which to produce the polysaccharide. Yields of purified material were so small, however, that this method was abandoned. When grown on solid gelatin or agar media results were much more consistent and the purified polysaccharides from each medium were identical. The agar medium was adopted for large scale work. This *Azotobacter* polysaccharide was purified by the method described for the *Rh. radicicolum* polysaccharide, which it resembled very closely in physical properties. Its content of bacterial protein was comparatively small and its purification was readily effected. A purified dried sample was a white asbestos-like mass, which dissolved in water forming a viscous clear solution $[\alpha]_D - 2^\circ$; ash, 8%; uronic anhydride 4%. Hydrolysis of the polysaccharide was effected with $N/2$ H_2SO_4

at 100° in about 10 hr. From the hydrolysate ($[\alpha]_D + 26°$), crystalline *d*-glucose in 87 % yield and the barium salt of a uronic acid in 3 % yield were isolated. The recognition of a small percentage of a uronic acid as a constituent of the *Azotobacter* polysaccharide molecule places this polysaccharide in the same class as the type II and III *Pneumococcus* polysaccharides. Further investigations of molecular structure will be reported later.

Experimental

I. Rhizobium radicicolum *polysaccharide*

The organism was supplied by Dr H. Nichol (*vide supra*). It remained quite active as a gum producer on suitable medium over a long period of subculturing. The basal culture medium was essentially that previously employed [Cooper & Preston, 1937] and had the following composition:

Lucerne-root extract agar (dissolved in 500 ml. of tap water): K_2HPO_4 0·5 g., $MgSO_4$, $7H_2O$ 0·20 g., NaCl 0·20 g., $MnCl_2$ 0·01 g., $FeCl_3$ 0·01 g., $CaCO_3$ 3·0 g., sucrose 20 g., asparagine 5 g.

Powdered lucerne root (5 g.) was boiled with distilled water (500 ml.) for 1 hr., allowed to stand overnight and filtered through cotton wool. This extract was added to 500 ml. of hot basal medium in which was dissolved powdered agar (18 g.). Transference of the medium in 20 ml. portions to boiling tubes was immediately carried out. Stock cultures of the organism were maintained on 5 ml. slopes.

Investigations on liquid media. Using the above basal salt medium, additional substances in varying amounts were added in an endeavour to study the factors influencing the production of polysaccharide. In general the medium was distributed in small conical flasks, inoculated from slopes and incubated for about 10 days. The mucilaginous growth was diluted with distilled water, filtered through cotton wool and the solution run into twice its volume of alcohol. The polysaccharide which usually rose to the surface, was isolated and examined. Production of polysaccharide from media containing varying concentrations of sucrose was first studied. Observations showed that a 5 % sucrose concentration gave maximum yields. Other constituents were now varied and the following conclusions were reached:

(*a*) Addition of a maximum amount of 1 % beet molasses was sufficient to produce growth and polysaccharide formation, the latter reaching a maximum in 10 days at 30°. If molasses was employed, the medium developed a very dark colour during sterilization and the polysaccharide formed was difficult to separate from pigmented material. Initial clarification of the molasses could readily be effected by charcoal but this treatment reduced considerably its power as a growth stimulator.

(*b*) Demerara and beet sugars, alcoholic extracts of peptone or molasses were active in producing growth, whilst malt extract, maple syrup and lecithin were without effect.

(*c*) Addition of 0·02 % of caffeine stimulated polysaccharide formation but it could not replace asparagine as a nitrogen source.

For larger scale work the following medium was employed: KH_2PO_4 2 g./l., $MgSO_4$, $7H_2O$ 0·2 g./l., NaCl 0·2 g./l., $MnCl_2$ 0·01 g./l., $FeCl_3$ 0·01 g./l., $CaCO_3$ 5·0 g./l., asparagine 1·0 g./l., caffeine 0·2 g./l., sucrose 50 g./., alcoholic extract of molasses 10 ml./l.

200 ml. amounts of this medium, distributed in 500 ml. conical flasks were steamed on 3 successive days and inoculated with 1 ml. of an active 48 hr. culture

of *Rh. radicicolum* (clover variety) growing in the same medium. The flasks were incubated at 30° for 10 days. The crude polysaccharide was precipitated by pouring the viscid solution into excess of ethyl alcohol (2 vol.) containing a few drops of calcium chloride solution. The gummy fibrous mass which rose to the surface was isolated, washed with absolute alcohol and ether and dried in a vacuum. It formed a stable white asbestos-like mass. Maximum yield of crude material, 300 mg./l. of medium.

Products made on liquid medium were grossly contaminated with protein material and ash (mainly phosphate). After acid hydrolysis the polysaccharides were shown to be of variable composition and the method was abandoned.

Production of the polysaccharide on a solid gelatin medium. A medium having the following composition was prepared: KH_2PO_4 1·0 g./l., $KHPO_4$ 1·0 g./l., NaCl 0·2 g./l., $MgSO_4$, $7H_2O$ 0·2 g./l., $CaCO_3$ 5·0 g./l., $Fe_2(SO_4)_3$ 0·01 g./l., $MnCl_2$ 0·01 g./l., $CaSO_4$ 0·01 g./l., asparagine 1·0 g./l., sucrose 40·0 g./l., gelatin 150·0 g./l.

It was dispersed in 200 ml. amounts in Roux bottles, steamed on three successive days and inoculated by means of small sterile pipettes with a 48 hr. culture of the organism. After incubation at 20° for 14 days the polysaccharide was washed off with distilled water, the solution filtered through kieselguhr and purified by the method described later. Yield of crude material 50 mg./Roux bottle. It readily dissolved in water forming a viscous clear solution, $[\alpha]_D -20°$ (*c*, 0·21). Ash, 2·0%. On hydrolysis with $N/2$ H_2SO_4 at 100° the following changes were observed: initial value $[\alpha]_D -20°$, 45 min. +5·0°, 70 min. +10°, 120 min. +12°, 220 min. +15° (equilibrium value).

Production of the polysaccharide on agar medium. Preliminary experiments were carried out on 20 ml. slopes in boiling tubes. After a suitable incubation period the mucilaginous growth was diluted with water, filtered through cotton wool and dropped into two volumes of alcohol. The precipitated polysaccharide was isolated in the usual manner and purified by alcoholic fractionation. It was identical with the polysaccharide grown on gelatin medium. $[\alpha]_D -18°$ (*c*, 0·5); ash 5·0%. On hydrolysis with $N/2$ H_2SO_4 at 100° the following changes were observed: initial value $[\alpha]_D -18°$, 100 min. +8°, 170 min. +15°, 220 min. +18° (equilibrium value). No mucic acid could be obtained after nitric acid oxidation of the polysaccharide.

Control experiments were carried out: (*a*) with Roux bottles of identical agar medium but uninoculated; (*b*) using similar Roux bottles inoculated with two nitrogen-fixing organisms, *B. ureae* (Beijerinck) and *B. trauffauttii*. These bottles were incubated and treated in a manner similar to that used for isolating the *Rh. radicicolum* polysaccharide. Although the two organisms grew well on the medium in no case was any polysaccharide obtained.

From these experiments it was apparent that contamination by agar was slight, and after a series of experiments to determine optimal conditions whereby the yield per Roux bottle was increased to 0·25 g. it was considered advantageous to use the following medium for large-scale production: (dissolved in 500 ml. of water) asparagine 1·0 g., K_2HPO_4 1·0 g., $MgSO_4$, $7H_2O$ 0·2 g., NaCl 0·2 g., $MnCl_2$ 0·01 g., $FeCl_3$ 0·01 g., sucrose 40·0 g., caffeine 0·2 g., agar 17·0 g., lucerne root extract 500 ml.

The organism was grown on this medium distributed in 200 ml. amounts in Roux bottles and incubated at 25° for 10 days. The heavy growth was removed by addition of a few ml. of distilled water and rubbing gently with a glass rod. The resultant viscous solution was filtered through glass wool, and the polysaccharide isolated as usual by alcoholic precipitation.

Purification and general properties of the polysaccharide. The material, re-suspended in water, formed a viscous opalescent colloidal solution which contained appreciable amounts (60 %) of bacterial "debris" in suspension. This "debris" was mainly of a protein nature and it could not be removed by the usual centrifugal methods. Attempts to clear the solution by digestion with trypsin, pepsin or papain were partially successful, but this method was abandoned on realization that commercial preparations of these enzymes contained variable but usually significant amounts of carbohydrate material.

Intensive drying of the crude polysaccharide followed by fractional alcoholic precipitation from aqueous solution removed the bulk of suspended material which was separated in the fractions isolated at low alcohol concentrations. This method was tedious and the following "kieselguhr filtration method" was more convenient and of general application. A small amount of kieselguhr was suspended in distilled water and flooded on to a large Büchner funnel. More kieselguhr was stirred into a very dilute solution of the polysaccharide which was filtered several times under slight vacuum through the prepared filter. The filtrate was evaporated *in vacuo* to a syrup, poured into excess alcohol and the precipitated polysaccharide isolated as usual. A typical sample had the following properties: $[\alpha]_D^{18^\circ} - 17^\circ$ (*c*, 0·5); acid equivalent (after precipitation from HCl), 1000; ash, 4·6 %; moisture, 10 %; uronic anhydride, 18·0 %; nitrogen (traces).

On hydrolysis the rotation changed from $[\alpha]_D - 15^\circ$ to $+22^\circ$ (when heated with N H_2SO_4 at 80° for 2 hr.). Crystalline *d*-glucose ($[\alpha]_D + 52·5^\circ$) was isolated in 67 % yield together with the barium salt of a uronic acid in 23 % yield (Ba, 16 %). This salt is being further investigated and may be an aldobionate.

Polysaccharide from the lucerne strain of Rhizobium radicicolum

According to Hopkins *et al.* [1930] the gum from the *Rh. radicicolum* (lucerne strain) contains much less glucuronic acid (4 %) than that from the clover strain which has (approx.) 20 % glucuronic acid. As we had available an improved culture medium, it was thought desirable to isolate the polysaccharide from the lucerne strain in order to ascertain whether, under conditions more advantageous to growth and metabolic activity of the organism, the polysaccharide would contain a higher percentage of uronic acid. Our observations confirmed those of Hopkins *et al.* in that "lucerne" polysaccharide from agar medium containing glucose had 4·4 % uronic anhydride and the "lucerne polysaccharide from agar medium containing sorbitol had 3·6 % uronic anhydride. Investigations are in progress to determine whether each different strain of the *Rh. radicicolum* produces a polysaccharide specific to that strain.

II. *Polysaccharide formation by* Azotobacter chroococcum

On liquid media. The organism was freshly isolated from soil at Edgbaston, Birmingham. It was particularly active as a gum-producer, for its synthetic powers did not diminish over a long period of subculturing. Investigation of polysaccharide formation in liquid media was continued and the properties of the materials isolated were compared with those of the polysaccharide grown on a solid medium. After a series of experiments during which the amounts and nature of both carbon and nitrogen sources were varied considerably, the following medium was found convenient and gave optimum yields: K_2HPO_4 0·5 g./l., $MgSO_4$, $7H_2O$ 0·2 g./l., NaCl 0·2 g./l., $CaSO_4$ 0·1 g./l., $FeCl_3$ (trace), sucrose 50·0 g./l., peptone 1·0 g./l., $CaCO_3$ 20 g./l.

Polysaccharide formation was poor when large volumes of medium were used and our results confirmed Anderson's observation [1933] that when growth did take place it was always accompanied by gum formation. Sorbitol (50 g./l.) was a good alternative to sucrose and in this case the metabolism solutions became highly viscous. The use of sorbitol minimizes any possible contamination from "laevan-forming" organisms [Cooper & Preston, 1935]. Yield of crude polysaccharide, 0·3 g./l. The products formed viscous suspensions in water which were too opaque for observation of optical rotation and very scanty amounts were obtained after purification. The medium was eventually abandoned.

On a gelatin medium. The medium used was essentially the same as that described for production of *Rh. radicicolum* polysaccharide. The method of growth, isolation and purification of the polysaccharide was also similar.

Properties of the polysaccharide. $[\alpha]_D - 2^\circ$ (*c*, 0·52) in water; ash, 3·1 %. Hydrolysis with $N/2$ H_2SO_4 (*c*, 0·52), $[\alpha]_D - 2^\circ$ (initial value), 0° (30 min.), +6° (70 min.), +10° (190 min.), +16° (280 min.), +20° (355 min.), +24° (690 min.) (equilibrium value). A naphthoresorcinol test for uronic acid was positive.

On an agar medium. Detailed accounts of the numerous investigations carried out in order to determine the best condition of growth are unnecessary. A medium of the following composition gave reliable results: (dissolved in 1 l. of 10 % yeast water) K_2HPO_4 0·8 g., KH_2PO_4 0·2 g., NaCl 0·2 g., $MgSO_4$, $7H_2O$ 0·2 g., $CaSO_4$ 0·1 g., $FeSO_4$ 0·01 g., $CaCO_3$ 5·0 g., sucrose 50 g., agar 17 g.

The medium was distributed in 200 ml. portions in Roux bottles, sterilized by steaming on three successive days and inoculated (using sterile pipettes) from an emulsion of a 48 hr. fluid culture. After incubation for 10 days at 25° the white viscous growth was washed off with a small amount of distilled water and the solution filtered through cotton wool into excess alcohol. The polysaccharide was readily precipitated in the form of a white fibrous mass which in appearance closely resembled the *Rh. radicicolum* polysaccharide, although the latter usually contained considerably larger quantities of bacterial protein. Purification of the polysaccharide was readily effected by filtration through kieselguhr in the manner previously described. The polysaccharide showed $[\alpha]_D - 2^\circ$ (*c*, 0·52). It dissolved rapidly in water to give a viscid clear solution which was neutral. Ash, 8·3 %; uronic anhydride, 4 %. Hydrolysis was carried out at 100° with $N/2$ H_2SO_4 and the following rotational changes were observed: initial value $[\alpha]_D - 6^\circ$ (*c*, 1·2), 60 min. −2°, 96 min. +2°, 138 min. +5°, 168 min. +7°, 210 min. +10°, 330 min. +17°, 510 min. +25°, 570 min. +26° (equilibrium value). From the hydrolysis solution crystalline glucose, $[\alpha]_D^{20^\circ} + 52{\cdot}3^\circ$ (*c*, 1·0), was isolated in 87 % yield together with a small amount of the barium salt of a uronic acid (3 % yield) which gave a positive napthoresorcinol test. Tests for ketose or pentose were negative and no mucic acid could be isolated from oxidation of the hydrolysate.

Summary

Suitable media for large scale preparation of the polysaccharides from *Rhizobium radicicolum* (clover strain) and from *Azotobacter chroococcum* have been described. The polysaccharides have been purified and their hydrolysis products examined. This *Azotobacter* polysaccharide contains glucose units (about 90 %) and a small amount of a uronic acid residue (3–4 %). In this respect and in physical properties the polysaccharide resembles that from *Rh. radicicolum* and both probably belong to the same class as the specific polysaccharides of *Pneumococcus* types II and III.

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