STARCHLIKE RADIATE CRYSTALS PRODUCED BY BACTERIUM MARGINATUM IN STARCH MEDIA[1]

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INTRODUCTION

Bacterium marginatum L. McC.[2] is a plant pathogene rather widely distributed in the United States and Canada, and it has also been reported from Europe. It causes a destructive disease on both the foliage and the corms of gladioli. This organism when grown on potato-dextrose agar or other suitable media produces in great abundance beautiful spherocrystals (fig. 1) which become blue when treated with iodine solution. In most cultures the crystals are 15 to 35 μ in diameter, but some of 140 μ have been found. They are apparently composed of needlelike parts radiating from a central point. Often there are two centers, and a double form results. (Fig. 1, B.) When broken by pressure on the cover glass they split into irregular triangular and needlelike parts.

In spite of the numerous radiating lines, the crystals are transparent. By polarized light they are faintly luminous, and the larger forms show a dark cross with the lines intersecting at right angles in the center. Some crystals show, in addition to the cross, several alternate light and dark concentric circles extending from near the center to the circumference, others have irregular or broken concentric light and dark markings, and still others show no trace of such markings. These concentric circles became invisible when a selenite plate was placed between the Nicols. Other crystals of normal appearance, but developed in a different medium, showed the dark cross less distinctly and none of the concentric marking. With a selenite plate these crystals showed two yellow and two blue-violet sectors. Still other crystals examined by C. L. Alsberg, of the Food Research Institute at Stanford University, Calif., showed "a very faint cross" and with a selenite plate between the Nicols "an alternate blue and yellow concentric-circle arrangement."

Schardinger [3] [4] discovered a thermophilic bacillus, *B. macerans*, which converted a considerable amount of the starch in a culture medium into a crystalline material which he first (1903) designated as "Krystallisierte Dextrine" and later (1909) as "Krystallisierte Amylodextrin." Other workers have studied these substances, which are now generally known as crystalline amyloses.

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[1] Received for publication Feb. 28, 1929; issued October, 1929.

[2] McCulloch, L. A LEAF AND CORM DISEASE OF GLADIOLI CAUSED BY BACTERIUM MARGINATUM. Jour. Agr. Research 29: 159–177, illus. 1924.

[3] Schardinger, F. ÜBER THERMOPHILE BAKTERIEN AUS VERSCHIEDENEN SPEISEN UND MILCH, SOWIE ÜBER EINIGE UNSETZUNGSPRODUKTE DERSELBEN IN KOHLENHYDRATHALTIGEN NÄHRLÖSUNGEN DARUNTER KRYSTALLISIERTE POLYSACCHARIDE (DEXTRINE) AUS STÄRKE. Ztschr. Untersuch. Nahr. u. Genussmtl. 6: 874. 1903.

[4] ——— UEBER DIE BILDUNG KRISTALLISIERTER, FEHLINGSCHE LÖSUNG NICHT REDUZIERENDER KÖRPER (POLYSACCHARIDE) AUS STÄRKE DURCH MIKROBIELLE TATIGKEIT. [VORLÄUFIGE MITTEILUNG AUS DER K. K. ALLGEMEINEN UNTERSUCHUNGSANSTALT FÜR LEBENSMITTEL IN WIEN.]. Centbl. Bakt. [etc.] (II) 22; 98–103, illus. 1909.

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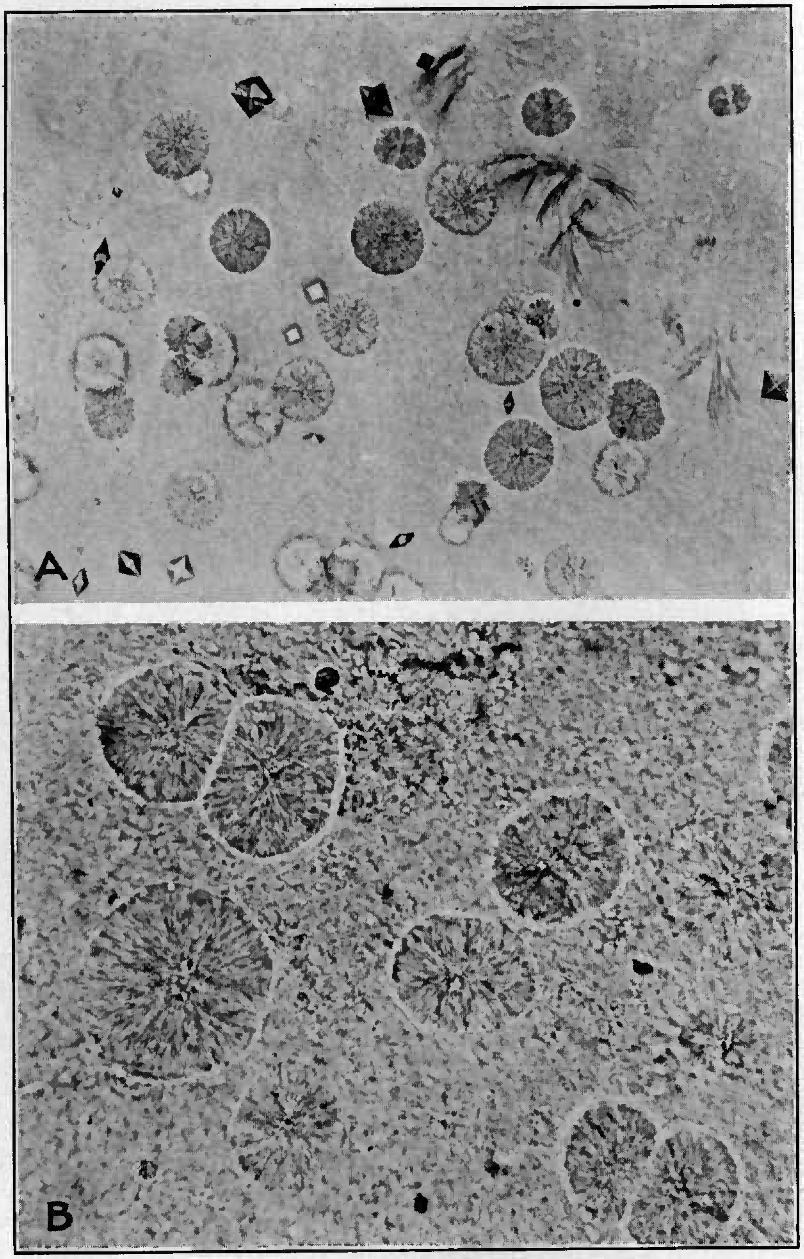

FIGURE 1.—A, Spherocrystals and also several rectangular crystals of calcium oxalate and tufts of slender, flexible crystals(?). × 250. B, Spherocrystals. × 500

The crystalline substances produced by *Bacillus macerans*, as described by Schardinger, are in form unlike those produced by *Bacterium marginatum*. However, Schardinger seems to have made microscopic examinations of his material only after various processes of filtering, neutralizing, and evaporation, which may have changed the form of the crystals. The morphological and cultural characters of *B. macerans* as given by Schardinger are very unlike those of *Bact. marginatum*.

Van de Sande-Bakhuyzen [5] pointed out that starch grains are built up of radiating needles. In a later paper [6] he demonstrated that under certain conditions spherocrystals are formed in an amylose solution which was obtained by grinding and then washing and centrifuging starch grains in cold water. The crystals so obtained are composed of several or numerous radiating needles. An alcoholic solution of iodine colors them brown to purple.

By heating a 10 per cent starch paste and then cooling it slowly, Beijerinck [7] obtained a crystalline deposit consisting of fine needles either isolated or in groups of various shapes. These he considered crystallized starch on account of their behavior toward diastase and chemical reagents.

CULTURE MEDIA

The crystals formed by *Bacterium marginatum* have been found only in culture media containing starch and a sugar or an alcohol. Potato agar with 2 per cent of dextrose [8] has been most commonly used, but a 2 per cent solution of pure starch in a synthetic solution has been successfully substituted for the potato broth. Saccharose, lactose, maltose, galactose, mannitol, and glycerol have each been successfully substituted for the dextrose.

The crystals form in either solid or liquid media. In solid media they are found in the medium, not in the layer of bacterial growth. In liquid media most of the crystals are found in the thick, almost viscid surface growth.

The crystals develop in the cultures during or just following an acid fermentation (without development of gas) and destruction of the sugar. Under favorable conditions the crystal formation, starting in the medium immediately adjoining the bacterial growth, proceeds into all parts. Crystals have been found in cultures on the third day after inoculation, and by the sixth or seventh day they are usually abundant in the upper half of the medium. They have been found in agar tubes 45 mm. below the bacterial growth and in plates 70 mm. from the nearest colony. They are apparently equally numerous (500 to 800 to the square millimeter of agar) in all parts of the culture. In agar cultures the crystals retain their structure and characteristic reaction with iodine solution in old (some 5 years old) dry cultures.

As the acid fermentation proceeds the slightly cloudy, opalescent potato-dextrose agar becomes more transparent. A cloudy line (due to closely packed, tiny, irregular crystals) usually divides the trans-

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[5] Van de Sande-Bakhuyzen, H. L. The structure of starch grains of wheat grown under constant conditions. Soc. Expt. Biol. and Med. Proc. 23: 302–305. 1926.

[6] ——— Crystallization of starch. Soc. Expt. Biol. and Med. Proc. 23: 506–507. 1926.

[7] Beijerinck, M. W. "Crystallized starch." K. Akad. Wetensch. Amsterdam, Proc. Sec. Sci. 18: 305–309, illus. 1916.

[8] Potato, 500 gm., sliced and steamed, in 1,000 c. c. distilled water, strained; 1½ per cent agar added; steamed again, filtered; 2 per cent dextrose added; tubed, and sterilized by autoclaving.

parent fermented part from the unfermented, the line advancing as the fermentation proceeds. (Fig. 2, A and B.) The spherocrystals are abundant in the cleared, fermented area; they are occasionally found in or just beyond the cloudy line, but are entirely lacking in the unfermented part. Tests made with Fehling's solution at the close of the fermentation period show that all the sugar has disappeared, while the starch content seems unchanged. The tests for starch have been made in only a crude way by adding iodine solution and comparing with checks. In most cultures there seems to be no difference in the starch reaction between cultures and checks. In others there is apparently less starch in the cultures. More exact methods will be necessary to decide how much starch, if any, has been changed. If peptone is added to the culture medium the production of the crystals is apparently hindered, but not prevented, and the sugar is less completely destroyed.

The exact conditions and media for the best production of these crystals have not yet been determined. The potato-dextrose agar is not always suitable. Sometimes there are variations in parallel cultures of the same strain of the bacteria grown on the same medium. One tube may have numerous crystals and others few or none. A few cultures have been observed with crystals in only certain areas. Sometimes an increased amount of sugar favored and at other times seemed to hinder crystal production.

The natural pH value of the potato-dextrose medium varies from 5.2 to 6.6. By adjustment, values from 4.8 to 7.9 have been made. While the bacteria grew on all of these, the values from 5.2 to 6.6 appeared most favorable. Regardless of the original pH of the medium, the first reaction to the bacterial growth is acid. The peak of the acid reaction is 4.2 to 4.4, after which there is a change toward the alkaline, sometimes reaching 8.4

The best results up to the present both for numbers and size of the crystals were secured with a soft agar medium of pH 5.4 (potassium biphosphate, 0.2 gm.; asparagine, 1.5 gm.; dextrose, 5 gm.; galactose, 5 gm.; potato starch, 10 gm.; water, 500 c. c.; agar, 2.5 gm.). A liquid medium of pH 5.2 (potassium biphosphate, 0.2 gm.; potassium chloride, 0.05 gm.; magnesium sulphate, 0.05 gm.; calcium sulphate, 0.01 gm.; asparagine, 3 gm.; dextrose, 20 gm.; corn starch, 20 gm.; trace of iron; water, 1,000 c. c.) induced a heavy surface growth of bacteria, and numerous crystals were present three weeks after inoculation. Three months later the crystals were comparatively scarce and irregular in shape but still reacted typically with iodine solution. Another agar of pH 6.6 (potassium nitrate, 0.04 gm.; dextrose, 4 gm.; soluble starch, 2 gm.; agar, 2 gm.; water, 200 c. c.) was not favorable for the bacterial growth, but some of the starchlike crystals were produced.

Cultures were grown at temperatures ranging from 25° to 30° C.

It occasionally happens that, due to some unknown condition, the bacterial activity is limited in media supposed to be favorable. In such cases little or no fermentation occurs and no starchlike crystals develop; or some fermentation may occur without the formation of crystals.

A rough type of colony that occasionally appears in plate cultures of the normally smooth-surfaced colonies of *Bacterium marginatum* also produces the starchlike crystals.

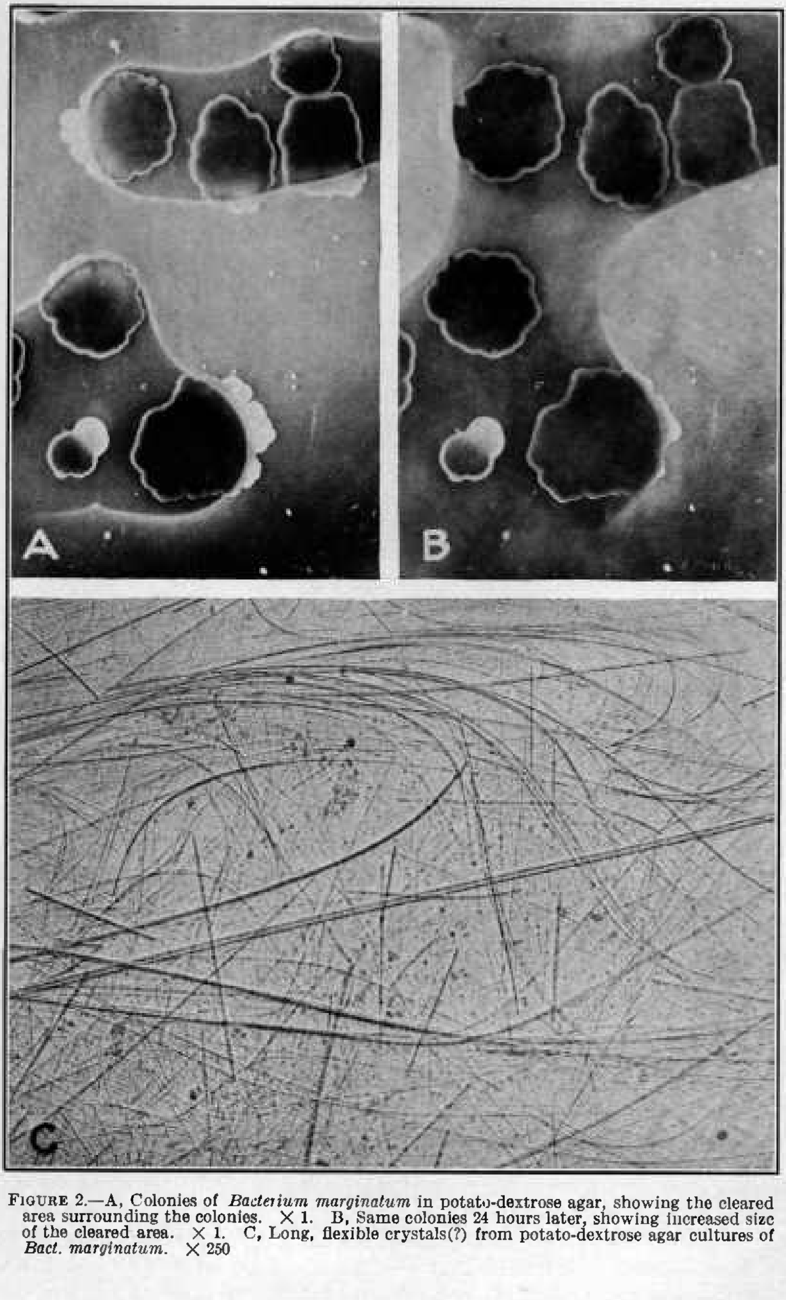

FIGURE 2.—A, Colonies of *Bacterium marginatum* in potato-dextrose agar, showing the cleared area surrounding the colonies. × 1. B, Same colonies 24 hours later, showing increased size of the cleared area. × 1. C, Long, flexible crystals(?) from potato-dextrose agar cultures of *Bact. marginatum.* × 250

All of the 20 or more strains of *Bacterium marginatum* used in the experiments have produced these starchlike crystals, but some strains have more readily and regularly than others produced crystals of good size. Some strains which formerly produced the crystals now fail to do so.

A number of other species of bacteria have been grown in parallel cultures with *Bacterium marginatum,* but none of them produced the type of crystal found so abundantly in the *Bacterium marginatum* cultures.

TEMPERATURE AND CHEMICAL TESTS

Because these crystals react like starch with iodine solution and are produced only in the presence of starch, it seems probable that they are some form of starch; but as no way has yet been found to separate the crystals from the agar of solid cultures or from the bacterial slime of liquid cultures, their exact chemical nature is unknown. In the following tests, agar containing typical crystals was crushed to a paste and the whole mass treated together.

The crystals do not dissolve in cold water, but after several days in it they become very transparent and sometimes invisible until they are stained with iodine. In water at 90° to 100° C. they dissolve quickly, but they withstand water at 80° for at least one hour. Alcohol, both ethyl and amyl, 50 to 95 per cent, has no effect on them in three hours. There is no effect from ether, chloroform, acetone, saturated solution of sodium phosphate, acetic acid (1, 10, 50, and 100 per cent), ammonia (concentrated), or 20 per cent picric acid. Alcohol (90 per cent) plus a small amount of sulphuric acid has no effect in two hours, but in 50 per cent sulphuric acid in alcohol the crystals fade away, without gas formation, in one minute or less. In iodine solution the crystals become pale blue to black, depending on the strength of the solution and also on the permeability of the surrounding media. Millon's reagent, cold for 15 minutes, has no effect, but when heated slightly the crystals dissolve without any trace of color reaction. In 5 per cent sodium hydroxide the crystals dissolve. After 20 hours in hydrogen dioxide the crystals are cracked and larger. After 12 hours in a strong solution of chloral hydrate there is no visible change in the structure of the crystals. In a 5 per cent diastase solution, at 35° to 37° C., the crystals disappear in one and one-half hours or less.

ASSOCIATED CRYSTALS

While the starchlike crystal is the most interesting crystal formed in cultures of *Bacterium marginatum,* there are several other types. Most frequently seen are those of calcium oxalate (fig. 1, A), either as small granules or as large 8-sided forms up to 75 μ in size. These occur not only in media suitable for starchlike crystals but also in beef agar, beef bouillon, plain potato broth, and 2 per cent peptone solution.

In potato-dextrose agar cultures there are found some extremely long and slender hyaline forms which were at first supposed to be crystals. There is one record of some that polarized light. It is more likely that they are some sort of gummy substance. The diameter varies from 0.5 to 1.5 μ, usually uniform throughout the whole length, which is 10 to 700 μ. In cross section they are not round, but square or rectangular, the side walls being definitely at right angles. The longer forms are very flexible. They will bend almost double but break over a sharp angle. Often a number of these slender forms seem to originate at a common center. (Fig. 1, A.) Pressure on the cover glass usually causes them to flatten, some of them to 10 or more times their original width, and occasionally irregular enlargements form at the ends. Parallel close-lying threads sometimes coalesce, and eventually with continued pressure these crystals (?) disappear, seeming to melt and leave no trace. No water or other mounting medium was used. In cultures 2 to 3 months old these crystals (?) were still flexible, but instead of flattening or dissolving under pressure, they broke into short pieces.

Besides the preceding types, other less characteristic crystals are found, mostly in old cultures. These are irregularly spherical forms resembling hailstones, frost crystals, and rough-branched coral. Hollow brittle spheres 1 to 1.5 mm. in diameter occasionally appear in milk cultures. Their walls seem composed of minute crystals and some amorphous material. They do not dissolve in water or in 10 per cent potassium hydroxide. Alcohol, chloroform, ether, ammonia, acetone, and xylol have no effect on them, but they dissolve in dilute acetic, hydrochloric, or sulphuric acid.

SUMMARY

This paper describes a starchlike crystal which forms in cultures of *Bacterium marginatum* when starch is a constituent of the medium. These spherocrystals, up to 140 μ in diameter, are produced in great numbers. They are composed of needlelike or threadlike parts radiating from the center. They become blue when treated with iodine solution, and by polarized light the larger crystals show dark cross marks.

The chemical nature of these crystals has not been determined, because as yet no way has been found to separate them from the culture medium.