INTRACELLULAR STARCH FORMATION IN CORYNEBACTERIA

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ABSTRACT

CARRIER, E. BERNARD (Louisiana State University, Baton Rouge, La.) AND C. S. McCLESKEY. Intracellular starch formation in cor-yne bacteria. J. Bacteriol. **83**:1029–1036. 1962.—*Corynebacterium tritici*, *C. striatum*, *C. renale*, and *C. pseudodiphtheriticum* produce an intracellular starch-like material when grown on native starches; glucose-1-phosphate, mono-, di-, and trisaccharides do not serve as substrates for intracellular starch formation. *C. pseudotuberculosis* and *C. kutscheri* produce intracellular starch from starch substrates and glucose-1-phosphate. *C. diphtheriae* produces starch from glucose-1-phosphate only.

Polysaccharide synthesis by bacteria appears to be a common, if not universal, occurrence, but bacterial synthesis of starch-like polysaccharides giving a blue color with iodine is a relatively rare phenomenon.

The production of iodophilic polysaccharide from glucose-1-phosphate has been observed in *Neisseria* (Hehre and Hamilton, 1946, 1948; Hehre, 1948), a mutant strain of *Escherichia coli* (Doudoroff et al., 1949), *Corynebacterium diphtheriae* and certain streptococci (Hehre, Carlson, and Neill, 1947; Carlson and Hehre, 1949), and a clostridium from pig caecum (Nasr and Baker, 1949). With none of these organisms could glucose be substituted for glucose-1-phosphate.

Reports of the production of iodophilic material from monosaccharides by bacteria have been made by Svartz (1930), Spray (1948), and Doetsch et al. (1957). Bacteria capable of producing starch-like material from disaccharides include *E. coli* (Monod and Torriani, 1948), rumen streptococci (Hobson and Mann, 1955; Crowley, 1955; Crowley and Jevons, 1955), *Neisseria* (Hehre and Hamilton, 1946, 1948; and Hehre, 1948), and a gram-negative anaerobic rod from the rumen of sheep (Doetsch et al., 1957).

Starches, glycogen, amylose, amylopectin, dextrins, maltotriose, and maltose were suitable substrates for the synthesis of iodophilic material by the rumen streptococci described by Hobson and Mann (1955), Crowley (1955), and Crowley and Jevons (1955). Crowley and Jevons concluded that, since starch was hydrolized to maltose, the synthesis of iodophilic material was mediated by the enzyme amylomaltase.

No reports have been found of bacteria which require, for starch synthesis, substrates exceeding two hexose units in length. This paper presents evidence that certain corynebacteria produce intracellular iodophilic starch-like material only from substrates consisting of relatively long α-1,4-linked glucosidic chains.

MATERIALS AND METHODS

A total of 44 strains, representing 24 of the aerobic species of the genus *Corynebacterium* described in Bergey's Manual (Breed, Murray, and Smith, 1957), were initially employed in the study of starch utilization. The species found to produce intracellular starch from one or more substrates are shown in Table 1.

Nutrient agar (Difco) was used as the basal medium for the qualitative determination of compounds utilized in the formation of intracellular starch, with the exception that the phosphorylated carbohydrates were tested in filter-sterilized yeast-extract medium (Hehre et al., 1947).

Sugars were added to nutrient agar in 1% concentrations; natural starches, Ramalin (Stein-Hall & Co., New York, N. Y.), maltoheptaose and Nägeli amylodexrins (Dexter French, Iowa State University) were added in 0.3% concentration. Sterilization was performed in an autoclave at 121 C for 15 min. Superlose (Stein-Hall & Co., New York, N. Y.), a highly purified amylose fraction of potato starch, and rice amylose were dissolved in cold 0.1 N NaOH and neutralized with 1 N HCl immediately before they were added to media; they partially retrograded when added to nutrient agar. Aqueous solutions of the Schardinger alpha- and beta-dextrins (Corn Products Co.,

TABLE 1. *Corynebacteria producing intracellular starch from one or more substrates*

Species	Source	Laboratory designation
C. diphtheriae type *ulcerans*	ATCC 9015	1 di
C. diphtheriae type *gravis*	G. T. Hauser*	1 di G
C. diphtheriae type *mitis*	G. T. Hauser	1 di M
C. diphtheriae type *intermedius*	G. T. Hauser	1 di I
C. striatum	ATCC 6940	4 st
C. pseudotuberculosis	ATCC 8666	5 pst
C. renale	ATCC 10845	9 re
C. kutscheri	ATCC 11035	10 ku
C. pseudodiphtheriticum	ATCC 6981	17 psd
C. tritici	ATCC 11403	39 tr
C. tritici	(CT101) M. P. Starr†	45 tr

* Louisiana State Board of Health.
† University of California.

Argo, Ill.) were filter-sterilized and added to sterile nutrient agar to give 1% concentrations. Petri plates were prepared with the various media, inoculated by the streak method, and incubated for 5 days at 37 C. Starch formation was tested by flooding the colonies with Gram's iodine solution.

The intracellular location of the starch-like material was determined by microscopic examination of cells suspended in Gram's iodine and by the treatment of heated and unheated washed cells with saliva.

Nutrient broth containing 0.3% potato starch was used as the medium for the quantitative determination of the utilization of this substrate. Bacteria were removed by centrifugation and the cell-free supernatants tested for starch by the method of McCready and Hassid (1943). The cells were washed three or four times in distilled water and adjusted to an optical density of 0.210 (Spectronic 20, 660 mμ). To 5 ml of the adjusted cell suspension were added 0.05 ml each of Gram's iodine and 6 N HCl. Several minutes were allowed for maximal color development, after which optical densities were again determined. An increase in optical density (above 0.210) was considered to be due to the intracellular starch-iodine complex.

Reducing compounds were determined on the cell-free supernatants by the method of Somogyi (1945) and Nelson (1944). Paper partition chromatography (McCleskey, 1953; Townsend, 1955) was used to detect carbohydrates.

Nutrient agar containing 0.3% potato starch was used to study the effect of phosphate concentration, pH, and growth temperature on the synthesis of intracellular starch. Inorganic phosphate concentration was varied from 0.5 to 3.0%, to give a tenfold ratio of phosphate to substrate.

The hydrogen ion concentrations employed varied from pH 5.5 to 8.5. Cultures were streaked on the various media and incubated at 4, 20, 30, 37, and 45 C. Cultures on nutrient potato starch agar served as controls. After 5 days of incubation, the plates were flooded with Gram's iodine and compared visually with the control plates.

Inorganic phosphate liberation from glucose-1-phosphate was determined by the method of Fiske and SubbaRow (1925). The medium employed was that of Hehre et al. (1947). The bacteria were removed by centrifugation, and the cell-free supernatant fluids were tested for inorganic phosphate.

The type of intracellular iodophilic polysaccharide produced from starch was determined by the method of Schoch (1945).

RESULTS AND DISCUSSION

On nutrient agar containing potato starch, colonies of certain of the corynebacteria stained dark blue when flooded with Gram's iodine solution (Fig. 1). A faint reddish color was usually observed at the periphery of the blue colonies and extended 3 to 4 mm out into the medium. The light areas indicate hydrolysis of the substrate starch by contaminants which appeared 4 to 5 days after the plates were inoculated. The colonies of corynebacteria in the areas of hydrolysis stained blue when treated with iodine, suggesting the intracellular location of the starch-like material.

Microscopic examination of cells treated with iodine showed from one to several large blue granules per cell. The treatment of heated and unheated washed cells with saliva gave additional proof that the starch-like material was located within the cells (Fig. 2). Cells which were heated and then treated with saliva became negative for starch after 25 min; unheated cells, treated in the same manner, remained positive for starch.

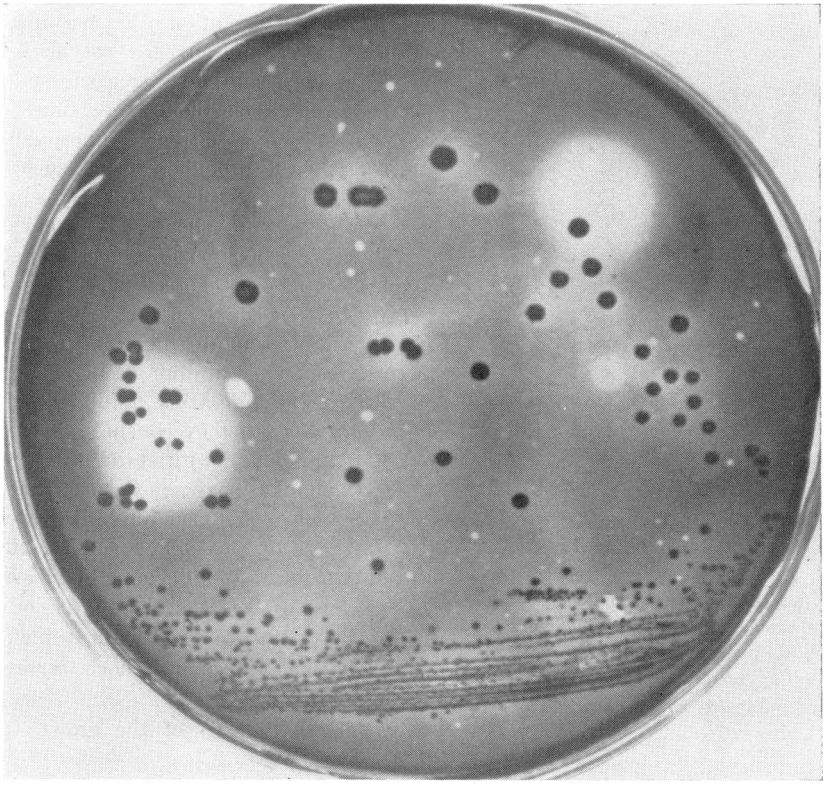

FIG. 1. *Colonies of Corynebacterium renale on starch agar, flooded with Gram's iodine. Light areas indicate hydrolysis caused by contaminants.*

The substrates utilized by the corynebacteria in the formation of intracellular starch are shown in Table 2. Glucose-1-phosphate was utilized for starch synthesis by all strains of *C. diphtheriae*, as reported by Hehre et al. (1947), and also by *C. pseudotuberculosis* and *C. kutscheri* (Carrier and McCleskey, 1961).

The polysaccharides produced from glucose-1-phosphate by *C. kutscheri* and the *intermedius* strain of *C. diphtheriae* gave a reddish-wine color; and those produced by *C. pseudotuberculosis* and *mitis*, *gravis*, and *ulcerans* strains of *C. diphtheriae* stained blue with iodine. This suggests that the polysaccharide of the former cultures is amylopectin or glycogen-like, similar to that produced from sucrose by *Neisseria* sp. (Hehre, 1948), and that of the latter is an amylose-type polysaccharide.

Corn starch, potato starch, rice amylose, Superlose, Ramalin, and, to a limited extent, dextrin were utilized by *C. striatum*, *C. pseudotuberculosis*, *C. renale*, *C. kutscheri*, *C. tritici*, and *C. pseudodiphtheriticum* in the formation of intracellular starch. In all these species, the intracellular polysaccharide produced from substrate starch was of the amylose type.

Only those corynebacteria producing iodophilic polysaccharide from glucose-1-phosphate released a significant amount of inorganic phosphate from that substrate (Fig. 3). *C. pseudotuberculosis* and *C. diphtheriae gravis* produced a blue-staining polysaccharide and released 0.0685 and 0.0705 g/100 ml, respectively, of inorganic phosphate after 117 hr. *C. kutscheri* produced a wine-red staining polysaccharide and released 0.0308 g/100 ml of inorganic phosphate. The results indicate that those species which do not produce starch from glucose-1-phosphate lack a transport mechanism for this compound, or else do not possess phosphorylase.

Hehre (1948) showed that by increasing the molecular concentration of inorganic phosphate to sixfold that of the substrate concentration, the action of phosphorylase obtained from a *Neisseria* sp. was inhibited. A tenfold ratio of inorganic phosphate to substrate starch failed to

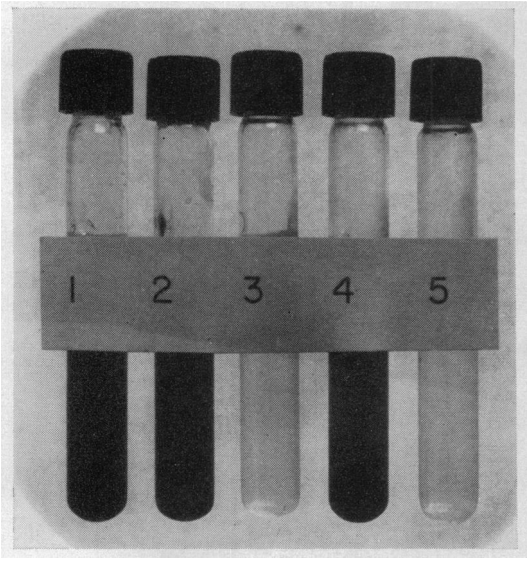

FIG. 2. *Effect of saliva on heated and unheated starch-grown washed cells of corynebacteria. Iodine was added after exposure to saliva for 25 min. Tube 1, unheated cells; tube 2, unheated cells plus saliva; tube 3, heated cells plus saliva; tube 4, heated cells; tube 5, starch plus saliva.*

inhibit the formation of intracellular starch from substrate starch by the corynebacteria tested. Although *C. pseudotuberculosis* and *C. kutscheri* contain phosphorylase, the failure of high concentrations of inorganic phosphate to inhibit starch synthesis from a starch substrate suggests that this is not the primary means by which these organisms synthesize intracellular starch from starch. Further evidence is suggested by the behavior of *C. kutscheri* in forming amylose from starch, but amylopectin-like (red-staining) material from glucose-1-phosphate.

Green and Stumpf (1947) noted that the Schardinger dextrins inhibited potato phosphorylase and they concluded that the dextrins were competing with starch for the same active site on the enzyme. Schardinger alpha- and beta-dextrin failed to inhibit intracellular starch synthesis by the corynebacteria, but the alpha-dextrin increased the amount of iodine required for formation of the blue starch-iodine complex, probably by sequestration of iodine (French, *personal communication*; Schoch, *personal communication*).

The initial pH of the growth medium and

TABLE 2. *Synthesis of intracellular starch-like polysaccharide from various substrates by corynebacteria**

Culture	Glucose-1-PO_4	Glucose-6-PO_4	Potato starch	Corn starch	Superlose†	Rice amylose	Ramalin‡	Dextrin	Glycogen	Phytoglycogen
C. diphtheriae (gravis)	+	−	−	−	−	−	−	−	−	−
C. diphtheriae (mitis)	+	−	−	−	−	−	−	−	−	−
C. diphtheriae (intermedius)	+	−	−	−	−	−	−	−	−	−
C. diphtheriae (ulcerans)	+	−	−	−	−	−	−	−	−	−
C. kutscheri	+	−	+	+	+	+	+	±	−	−
C. pseudotuberculosis	+	−	+	+	+	+	+	±	−	−
C. pseudo-diphtheriticum	−	−	+	+	+	+	+	±	−	−
C. renale	−	−	+	+	+	+	+	±	−	−
C. striatum	−	−	+	+	+	+	+	±	−	−
C. tritici	−	−	+	+	+	+	+	±	−	−

* All cultures were negative on glucose, fructose, maltose, sucrose, lactose, cellobiose, galactose, raffinose, melezitose, inulin, Schardinger alpha- and beta-dextrin, dextran, beta-limit dextrin, maltoheptaose, and Nägeli amylodextrin. Symbols: + indicates production of intracellular starch; ± indicates slight or doubtful production of intracellular starch; − indicates no production of intracellular starch.

† Amylose from potato.

‡ Amylopectin from potato.

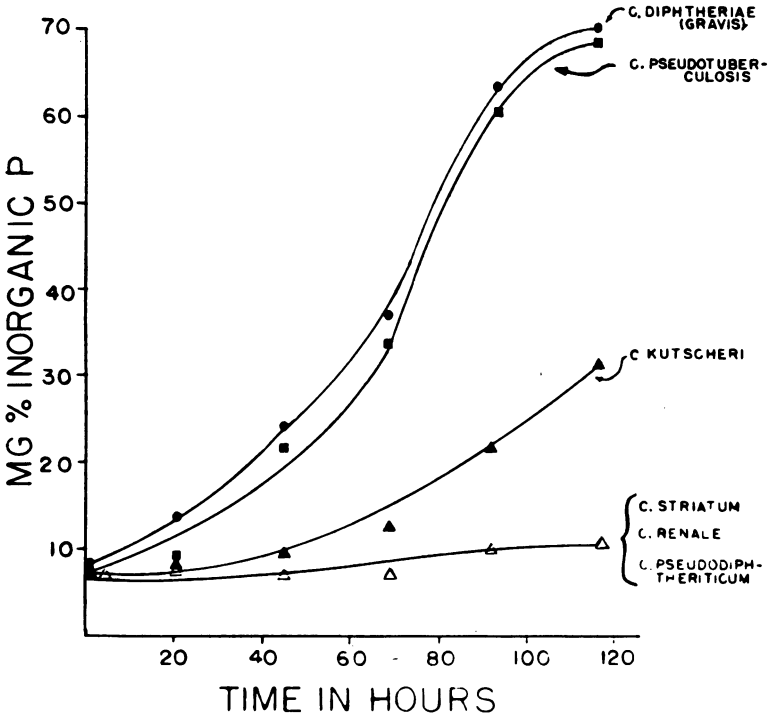

FIG. 3. *Phosphate released from glucose-1-phosphate by corynebacteria.*

TABLE 3. *Effect of initial pH and growth temperature on the synthesis of intracellular polysaccharide from potato starch*

Culture number	pH						Temperature (C)				
	5.5	6.0	6.5	7.5	8.0	8.5	4	20	30	37	45
4 st	+*	+	+	+	+	+		+	+	+	(+)
5 pst	(±)	+	+	+	+	+		+	+	+	(+)
9 re	(±)	+	+	+	+	+		+	+	+	(+)
10 ku	(±)	+	+	(+)	(+)	(+)		+	+	+	NG
17 psd	NG	NG	(+)	+	+	(+)		+	+	+	NG
39 tr	(±)	(±)	±	+	±	(±)	+	+	+	NG	NG
45 tr	(±)	(±)	±	+	±	(±)	+	+	+	NG	NG

* Parentheses indicate moderate growth inhibition; NG indicates no growth; + indicates production of intracellular starch; ± indicates slight or doubtful production of intracellular starch.

growth temperature had little effect on synthesis of intracellular starch; starch was formed at all temperatures and at all hydrogen ion concentrations allowing good growth of the organisms (Table 3).

Earlier reports of the synthesis of iodophilic polysaccharides by bacteria listed sugar phosphates, maltose, sucrose, and pentoses as satisfactory substrates. None of these seems to be a primary intermediate in the formation of intracellular starch by the corynebacteria grown in the presence of starch (Table 2). To test the possibility that degradation products of starch, other than those included in Table 2, may be intermediates in the formation of intracellular starch, chromatographic examinations, determinations of reducing compounds, and measurements of substrate and intracellular starch were

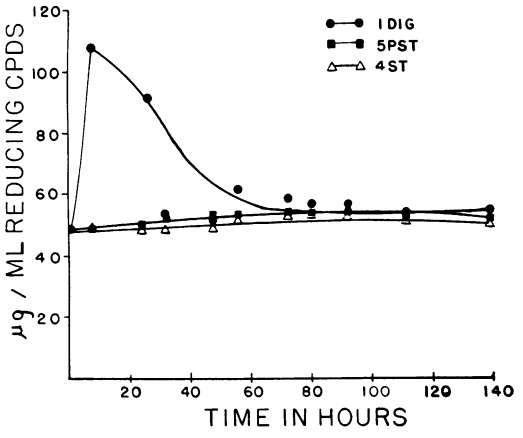

FIG. 4. *Reducing compounds produced in potato starch broth by corynebacteria. 1 di G, Corynebacterium diphtheriae; 4 st, C. striatum; 5 pst, C. pseudotuberculosis. None of the corynebacteria which produced intracellular starch from starch substrate produced reducing compounds from starch.*

made on the culture fluids at intervals throughout the incubation period. Since *C. diphtheriae gravis* possesses an amylase and does not form intracellular starch from potato starch, it was included in these studies as a control. *C. tritici* was not included since it produced intracellular starch only feebly from substrate starch.

The chromatograms revealed no carbohydrates in the mobile phase, except with *C. diphtheriae*. The results of tests for reducing substances are presented in Fig. 4. None of the species producing intracellular starch from the starch substrate showed significant increases in reducing substances.

The changes in substrate starch, intracellular starch, and reducing compounds effected by four species of corynebacteria which produce intracellular starch are shown in Fig. 5. In each instance, the decrease in substrate starch was accompanied by an increase in intracellular starch, without significant changes in the reducing value of the medium.

Though tests with culture supernatants and concentrated cell suspensions revealed no evidence of amylase activity, either by increase in reducing substances or by chromatography, the supernatants of sonic-treated cultures actively degraded starch. Intracellular starch usually decreased in cultures after depletion of the starch substrate.

The disappearance of substrate starch and the appearance of intracellular starch, without the occurrence of reducing sugars, suggests that possibly glucose or other fragments are transferred from the ends of the linear components of starch into the cell where new starch molecules are formed, or else the intact amylose molecule is transported directly into the cell. If the former hypothesis is true, the mechanism of intracellular starch synthesis appears to be by a "transglycosylase" (Hehre, 1951), unique in that it requires as substrate relatively long chains of α-1,4-linked glucosidic units. The failure to utilize glucose, glucose-containing di- and trisaccharides, beta-limit dextrin, phytoglycogen, glycogen, hepataose, and Nägeli amylodextrin suggests a requirement for chains longer than 20 glucose units. Since the only satisfactory substrate for starch synthesis was one giving blue color with iodine, and the full blue color depends on a chain length of 30 to 35 glucose units (Swanson, 1948), perhaps the chains must be at least 30 glucose units in length.

Our reluctance to accept the hypothesis that amylose molecules may be transported through the membrane of the bacterial cell led us (Carrier and McCleskey, 1961) to tentatively accept the first hypothesis, that the synthesis is mediated by a "transglycosylase" (Hehre, 1951). Using the precedent of nomenclature for amylosucrase (Hehre, Hamilton, and Carlson, 1949) and amylomaltase (Monod and Torriani, 1948), the descriptive term "amyloamylase" was applied to this enzyme which requires long chains of α-1,4-linked glucosidic units as substrate for the formation of intracellular starch.

The possibility that amylose may be transported directly into the cell has not been disproved, and the following observations appear to support this view. All the cultures which produce intracellular starch from substrate starch produce amylose; no amylopectin has been detected. Only those substrates which stain blue with iodine result in the formation of blue-staining intracellular polysaccharide. The reddish zone observed around colonies on starch agar may be due to the taking up of the amylose fraction of starch, leaving the red-staining amylopectin. The formation of intracellular amylose from Ramalin (a commercial amylopectin) may be due to the presence of a small amount (about 5%) of amylose in that product. Whether these organ-

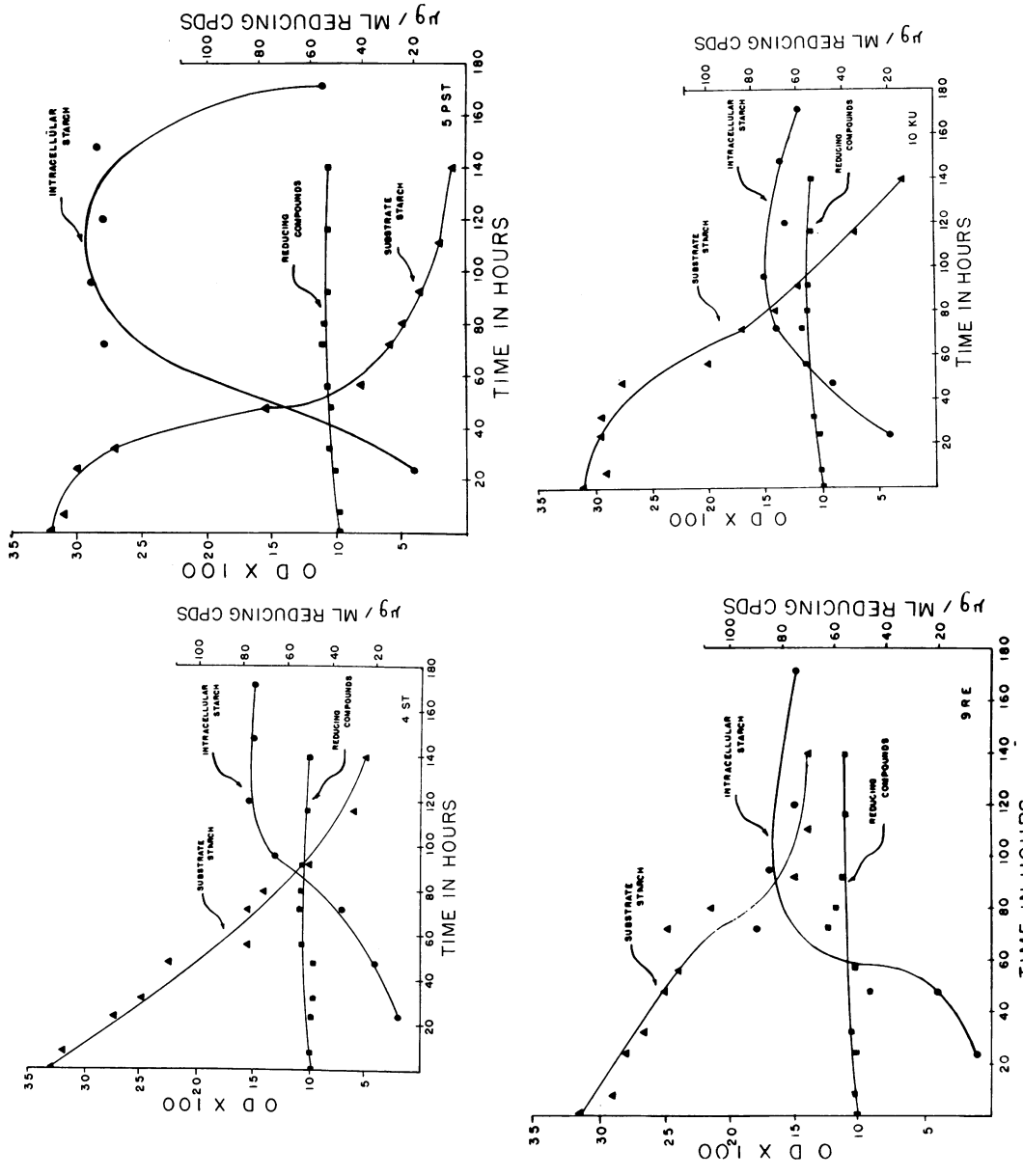

FIG. 5. *Utilization of substrate starch, intracellular starch formation, and reducing substances produced by corynebacteria, Upper left, Corynebacterium striatum; upper right, C. pseudotuberculosis; lower left, C. renale; lower right, C. kutscheri.*

isms synthesize starch with a transglycosylase or accumulate it by means of a permease, they appear to possess characteristics unusual among the bacteria.

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LITERATURE CITED

BREED, R. S., E. G. D. MURRAY, AND N. R. SMITH. 1957. Bergey's manual of determinative bacteriology. 7th ed. The Williams and Wilkins Co., Baltimore.

CARLSON, A. S., AND E. J. HEHRE. 1949. Composition of the starch synthesized from glucose-1-phosphate by diphtheria bacilli. J. Biol. Chem. **177**:281–293.

CARRIER, E. B., AND C. S. MCCLESKEY. 1961. Synthesis of starch-like material by corynebacteria. Bacteriol. Proc., p. 61.

CROWLEY, N. 1955. The action of streptococcal amylase in relation to the synthesis of an amylosaccharide by amylolytic strains of *Streptococcus pyogenes*. J. Gen. Microbiol. **13**:218–225.

CROWLEY, N., AND M. P. JEVONS. 1955. The formation of a starch-like polysaccharide from maltose by strains of *Streptococcus pyogenes*. J. Gen. Microbiol. **13**:226–234.

DOETSCH, R. N., B. H. HOWARD, S. O. MANN, AND A. E. OXFORD. 1957. Physiological factors in the production of an iodophilic polysaccharide from pentose by a sheep rumen bacterium. J. Gen. Microbiol. **16**:156–168.

DOUDOROFF, M., W. Z. HASSID, E. W. PUTMAN, A. L. POTTER, AND J. LEDERBERG. 1949. Direct utilization of maltose by *Escherichia coli*. J. Biol. Chem. **179**:921–934.

FISKE, C. H., AND Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**:375–400.

GREEN, D. E., AND P. K. STUMPF. 1942. Starch phosphorylase of potato. J. Biol. Chem. **142**:355–366.

HEHRE, E. J. 1948. The biological synthesis of polysaccharides. Trans. N. Y. Acad. Sci. **10**:188–198.

HEHRE, E. J. 1951. Enzymic synthesis of polysaccharides: a biological type of polymerization, p. 297–337. *In* S. P. Colowick and N. O. Kaplan [ed.], Advances in enzymology, vol. 2. Interscience Publishers, Inc., New York.

HEHRE, E. J., A. S. CARLSON, AND J. M. NEILL. 1947. Production of starch-like material from glucose-1-phosphate by diphtheria bacilli. Science **106**:523–524.

HEHRE, E. J., AND D. M. HAMILTON. 1946. Bacterial synthesis of an amylopectin-like polysaccharide from sucrose. J. Biol. Chem. **166**:777–778.

HEHRE, E. J., AND D. M. HAMILTON. 1948. The conversion of sucrose to a polysaccharide of the starch-glycogen class by Neisseria from the pharynx. J. Bacteriol. **55**:197–208.

HEHRE, E. J., D. M. HAMILTON, AND A. S. CARLSON. 1949. Synthesis of a polysaccharide of the starch-glycogen class from sucrose by a cell-free, bacterial enzyme system (amylosucrase). J. Biol. Chem. **177**:267–279.

HOBSON, P. N., AND S. O. MANN. 1955. Some factors affecting the formation of iodophilic polysaccharide in group D streptococci from the rumen. J. Gen. Microbiol. **13**:420–435.

MCCLESKEY, C. S. 1953. Paper partition chromatography with simplified apparatus and procedure. Proc. Louisiana Acad. Sci. **16**:31–32.

MCCREADY, R. M., AND W. Z. HASSID. 1943. The separation and quantitative estimation of amylose and amylopectin in potato starch. J. Am. Chem. Soc. **65**:1154–1157.

MONOD, J. M., AND A. M. TORRIANI. 1948. Syntheses d'un polysaccharide du type amidon aux depens du maltose, en presence d'un extrait enzymatique d'origine bacterienne. Compt. rend. **227**:240–242.

NASR, H., AND F. BAKER. 1949. Microbial synthesis of iodophilic polysaccharide by a clostridium from the caecum of the pig. Nature **164**:745.

NELSON, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. J. Biol. Chem. **153**:375–380

SCHOCH, T. J. 1945. The fractionation of starch. Advances in Carbohydrate Chem. **1**:247–275.

SOMOGYI, M. 1945. A new reagent for the determition of sugars. J. Biol. Chem. **160**:61–67.

SPRAY, R. S. 1948. The granulose reaction of certain anaerobes of the "butyric" group. J. Bacteriol. **55**:79–84.

SVARTZ, N. 1930. Pure cultures of *Clostridium butyricum iodophilum* from human feces. J. Infectious Diseases **47**:138–150.

SWANSON, M. J. 1948. Studies on the structure of polysaccharides. IV. Relation of the iodine color to the structure. J. Biol. Chem. **172**:825–837.

TOWNSEND, A. M. B. 1955. The dissimilation of levulose by *Leuconostoc mesenteroides*. M.S. Thesis, Louisiana State University, Baton Rouge, La.