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Physiological Factors in the Production of an Iodophilic Polysaccharide from Pentose by a Sheep Rumen Bacterium

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SUMMARY: A Gram-negative, non-flagellated, asporogenous, anaerobic, iodophilic, curved rod ($2.5 \mu \times 0.5 \mu$) was isolated from the rumen of a sheep and designated *Bacteroides amylogenes* n.sp. This organism transforms a number of carbohydrates including pentoses to intracellular iodophilic polysaccharide which has been isolated and shown to be a polymer of glucose. Resting suspensions of this organism utilize this iodophilic material as a reserve substance under a variety of conditions. *Bacteroides amylogenes* grows optimally *in vitro* when bicarbonate and rumen liquor are included in the medium. On physiological and morphological grounds it is considered to be an authentic rumen bacterium. The chief fermentation product from xylose is butyric acid.

Recent investigations by Robinson, Doetsch, Sirotnak & Shaw (1955), Mann & Oxford (1955), Gibbons, Doetsch & Shaw (1955), and Hobson & Mann (1955) revealed that authentic rumen bacteria may produce intracellular iodophilic polysaccharide from carbohydrate substrates. Earlier references dealing with 'starch' or 'glycogen' formation in bacteria of avian or mammalian intestines are given in Hobson & Mann (1955). Heretofore there has been but one report concerning iodophilic polysaccharide formation from D (+) xylose by mixed suspensions of bovine rumen bacteria (Gibbons *et al.* 1955). It was shown that a polysaccharide 'ISS' (an abbreviation for iodine staining substance), composed solely of glucose, was produced from either D (+) glucose or D (+) xylose by a mixed rumen bacterial flora, irrespective of the number of different 'species' that might be present.

The present paper studies the physiological factors which govern this interesting polymerization phenomenon when a pure culture of a Gram-negative sheep rumen bacterium, not previously described, is used. In view of this, morphological, physiological and nutritional data are given for taxonomic characterization of the organism. Although on morphological grounds alone (see organism no. 9 (fig. 21) of Moir & Masson, 1952, which resembles it) it may well be a rumen bacterium, the authenticity of this isolate is judged mainly in the physiological sense, i.e. it is obligately anaerobic; grows only over a narrow temperature range near 39°; requires a factor(s) found in rumen liquor for optimal *in vitro* growth; and forms ISS and end products normally associated with rumen liquor.

METHODS

Isolation of xylose-fermenting bacteria

Rumen liquor samples were obtained from fistulated sheep no. 879 (Eadie & Oxford, 1955). This animal was fed 750 g. hay and 450 g. concentrates/day. Rumen liquor samples were taken *c.* 3 hr. after morning feeding. The culture technique of Hungate (1950) was used, but with the dilution blanks devised by Doetsch, Robinson & Shaw (1952). D (+) Xylose (substituted for D (+) glucose and cellobiose) cysteine hydrochloride and Na₂CO₃ were sterilized together by Seitz filtration and added aseptically to the autoclaved rumen liquor + mineral salts agar to give final concentrations (w/v) of 1, 0.1 and 0.4 %, respectively. Roll tubes were incubated at 39° and colonies selected from 10⁻⁷ or 10⁻⁸ dilutions after 48–72 hr. Colonies were subcultured only when microscopic examination of a wet mount stained with Lugol's iodine solution revealed a purple or blue coloration within the cells (Hobson & Mann, 1955). One isolate (4.13) was selected from a collection of xylose-fermenting rumen bacteria for intensive study. Conventional pure culture techniques were used for Gram's staining method, capsule stain, granule stain, indole, H₂S and catalase production, and optimum growth temperature. Motility determinations were made in sealed capillary tubes, and flagella stains were made by the methods of Fisher & Conn (1942), Leifson (1951) and Plimmer & Paine (1921).

Range of compounds transformed to iodophilic polysaccharide

Most of the compounds studied (Table 1) were sterilized by Seitz filtration and substituted in final concentration of 1 % (w/v) for the D (+) xylose in the isolation medium. Agar slopes were streaked and stabbed with one loopful of a 24 hr. culture of organism 4.13 grown on xylose-rumen liquor + mineral salts agar (hereafter referred to as 'X agar'). Controls were X agar itself and the medium without added D (+) xylose. As soon as 'fair' (1+) growth appeared, the organism was subcultured to a fresh tube of the same medium to obviate 'carry over' effects. In no instance did growth appear in the medium without carbohydrate. Density of growth was estimated as 'fair' (1+) to 'luxuriant' (4+). Immediately following subculture the initial slope was flooded with Lugol's iodine solution and the colour noted. It was considered necessary to do so at the earliest possible moment after growth appeared, since the iodophilic polysaccharide is a reserve substance and is utilized upon continued incubation. Acid, but not necessarily gas formation was detected in this study, since carbonate (which appeared essential for optimal growth) liberated as CO₂ by fermentation acids split the agar and produced frothiness in the syneresis water.

Nutritional requirements

Several workers (Hungate, 1950; McNeill, Doetsch & Shaw, 1954; Bryant & Doetsch, 1954*a*) have shown that factors(s) found in rumen liquor are essential for *in vitro* cultivation of many authentic rumen bacteria. In addition to studying this point with isolate 4.13, the effect of bicarbonate on growth was

Table 1. *Growth and 'starch' production by Bacteroides amylogenes on various substrates*

The concentration of substrate in the agar medium was 1 % (w/v) unless otherwise stated; it was sterilized separately by Seitz filtration of a concentrated aqueous solution except in the case of polysaccharides of high molecular weight when sterilization was by intermittent steaming. Analar carbohydrates were used wherever possible; the source of the rarer sugars is indicated below.

Substrate	Amount of bacterial growth in (n) days	Iodophilic reaction of growth
Aldopentoses		
D (+) Xylose	4 + (1)	+
L (-) Xylose (L. Light)	0 (8)	0
D (-) Arabinose (L. Light)	1 + (1)	+
L (+) Arabinose (Kerfoot)	4 + (1)	+
D (+) Ribose (L. Light)	1 + (2)	+
D (+) Lyxose (L. Light)	0 (5)	0
Methylaldopentoses		
L (+) Rhamnose	4 + (1)	+
L (-) Fucose (L. Light)	4 + (1)	+
Aldohexoses		
D (+) Glucose	4 + (1)	+
D (+) Mannose (recrystallized from ethanol and methanol)	4 + (1)	+
D (+) Galactose	4 + (1)	+
Ketohexoses		
D (-) Fructose	4 + (1)	+
Sorbose	0 (8)	0
Sugar alcohols		
Glycerol	1 + (4)	±
Adonitol (G. T. Gurr)	0 (8)	0
Mannitol	0 (6)	0
Sorbitol	0 (6)	0
Dulcitol	0 (8)	0
Inositol	0 (4)	0
Aminosugar		
Glucosamine hydrochloride	4 + (2)	+
Hexose phosphates (0.5 %, w/v)		
Glucose-1-phosphate (K salt)	1 + (5)	+
Glucose-6-phosphate (K salt)	1 + (2)	+
Disaccharides		
Sucrose	4 + (1)	+
Maltose	4 + (1)	+
Lactose	1 + (4)	+
Cellobiose	4 + (1)	+
Trehalose (G. T. Gurr)	4 + (1)	+
Melibiose (L. Light)	4 + (1)	+
Trisaccharide		
Raffinose (Kerfoot)	4 + (1)	+
Glycosides		
α-Methylglucoside (B.D.H.)	0 (8)	0
Salicin	4 + (1)	+
Aesculin (G. T. Gurr)	4 + (1)	+
Pentosans		
Soluble wheat flour pentosan entirely free from hexosan; (contains xylose and arabinose units, Perlin, 1951)	3 + (1)	+
Soluble xylan of <i>Rhodymenia palmata</i> (Barry & Dillon, 1940)	1 + (2)	+

Table 1 (cont.)

Hexosans		
Inulin (Kerfoot)	4+(1)	+
Dextrin (Kerfoot)	1+(1)	+
Glycogen	0 (6)	0
Amylose, (0.5 %, w/v)	0 (6)	0
Holotrich starch (amylopectin) (Forsyth & Hirst, 1953)	0 (6)	0
0.5 % (w/v)		
Soluble starch (B.D.H.)	0 (6)	0
Cotton cellulose (finely divided, 0.2 %, w/v)	0 (12)	0
<i>Bacteroides amylogenes</i> 'starch'	0 (6)	0

Organic acids

The organism did not utilize any of the following: glucuronate, pyruvate, lactate, fumarate, succinate, citrate.

investigated. Substitutions for 40 % (w/v) rumen liquor in X agar (all given as % (w/v) 'Bacto' products) were made as follows: (1) 0.5 % Casitone; (2) 0.5 % Casitone + 0.5 % yeast extract; (3) 0.5 % yeast extract; (4) 0.5 % Tryptose; (5) 0.5 % yeast extract + 0.5 % peptone; (6) same as (1) but 0.066 M-phosphate buffer (pH 7.0) added aseptically (30 ml. in 50 ml. medium) without Na_2CO_3 , and tubed under N_2 ; (7) X agar without Na_2CO_3 and tubed under N_2 ; (8) a defined medium composed of the following per 100 ml.: calcium-D-pantothenate, 0.1 mg.; nicotinic acid, 0.25 mg.; pteroylglutamic acid, 0.001 mg.; thiamine hydrochloride, *p*-aminobenzoic acid, pyridoxine hydrochloride and riboflavin, 0.1 mg. each; inositol, choline chloride, nicotinamide, uracil, guanine hydrochloride, adenine sulphate and xanthine hydrochloride 0.5 mg. each; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.15 mg. each; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.014 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.056 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0034 g.; *n*-valeric acid, 5.0 mg.; *iso*-valeric acid, 2.5 mg.; sodium acetate, 0.41 g., sodium citrate $\cdot 2\text{H}_2\text{O}$, 0.2 g., NH_4Cl , 0.152 g., resazurin, 0.5 ml. of a 0.1 % (w/v) aqueous solution; agar (Gurr) 1.5 g., D (+) xylose, 1.0 g.; cysteine hydrochloride, 0.1 g.; Na_2CO_3 , 0.4 g. The latter three compounds were Seitz filtered and added aseptically to the medium which was then tubed under CO_2 ; (9) same as (8) but with 0.5 % each of yeast extract and proteose-peptone added. The density of growth was compared with that on X agar after 24 and 48 hr. of incubation at 39°, and for longer periods when necessary. Four serial subcultures were made on each medium supporting growth of isolate 4.13 before the observation was recorded.

Factors influencing the utilization of iodophilic polysaccharide

The growth of isolate 4.13 from 35 tubes of X agar (44 hr. at 39°) was washed off with 0.066 M-phosphate buffer (pH 7.0). It was found necessary to grow the organism on slopes of solid medium after repeated failure of attempts to obtain good growth quickly in liquid media or on solid medium in Roux bottles. The organisms were washed twice in buffer (no reducing sugar in supernatant) and the optical density adjusted to 0.78 at 640 m μ . (EEL filter no. 205) with the EEL (Evans Electroselenium Ltd., Harlow, Essex) colorimeter. These suspensions had a protein nitrogen content of usually about

0.28 mg./ml. Suspensions of organisms, adjuncts and buffer were made to a volume of 3 ml. in small rubber-stoppered test tubes and incubated under the appropriate O_2 -free gas in a 39° water bath. Table 2 is a protocol of the various factors investigated. After 1, 3 and 5 hr. incubation 0.25 ml. samples were removed from each tube and the organisms sedimented with an M.S.E. angle centrifuge. The sediment was washed once in buffer and the organisms suspended in a final volume of 2 ml. One-tenth ml. iodine solution (aqueous solution of 2% (w/v) KI + 0.2% (w/v) I_2) was added with thorough mixing, and the optical density read on the EEL colorimeter against a boiled-cell control arbitrarily set at O.D. = 1.00. The rate of disappearance of the blue colour was taken as a measure of polysaccharide utilization and the limiting optical density upon depletion was between 0.5 and 0.6.

End products of xylose fermentation

Several workers (Heald, 1952; Doetsch, Robinson, Brown & Shaw, 1953; Howard, 1955) have observed that mixed suspensions of rumen bacteria do not produce lactic acid from pentoses. To determine whether this held for isolate 4.13, and to note whether fatty acids characteristic of rumen liquor were produced from D (+) xylose fermentation, a liquid medium was devised which allowed fairly good if slow growth. The medium had the following composition: 0.5% (w/v) yeast extract, 0.5% (w/v) proteose-peptone, 10% (v/v) strained rumen liquor, 7.5% (v/v) of each of Hungate's mineral salt solutions, 0.1% (v/v) of a 0.1% (w/v) aqueous resazurin solution, 1.0% (w/v) D (+) xylose, 0.1% (w/v) cysteine hydrochloride, and 0.4% (w/v) Na_2CO_3 . The latter three compounds were sterilized by Seitz filtration and added aseptically to the autoclaved medium. Inoculum was one ml. of the complete medium used to wash off the growth from a 24 hr. culture of isolate 4.13 on X agar. Duplicate flasks (150 ml. volume filled two-thirds full) were set up, one incubated for a week at 39° and the other stored at 2° . At the end of the incubation period residual carbohydrate was determined by the method of Somogyi (1945), lactic acid by the Barker & Summerson (1941) method, and fatty acids by the method of James & Martin (1952).

Purification and characterization of iodophilic polysaccharide

Growth from 500 slope cultures of isolate 4.13 grown for 44 hr. on X agar at 39° was washed off with 0.066 M-phosphate buffer (pH 7.0) and twice filtered through 1 in. layers of closely packed glass wool. The organisms were washed twice in buffer and suspended in a minimal (1–3 ml.) amount of buffer. Reducing sugars were absent from the supernatant fluids of these washings. The resulting bacterial paste was kept at -20° until the extraction process was begun. The method used was that of Hobson & Mann (1955).

Sixteen g. cell paste (wet wt.) were ground in a cold mortar with 32 g. cold, acid-washed, acetone-extracted carborundum (grade F) [Griffin & Tatlock, Ltd., London] for 15 min. and the mass twice extracted with 33% (w/v) chloral hydrate at 80° . The extract was filtered through a sintered glass filter

(grade 5) and the filtrate slowly added with stirring to 4 vol. cold acetone. After standing 24 hr. at 2° the precipitated polysaccharide was collected by centrifugation and dried by trituration first with acetone and then with ether (yield 320 mg.). One hundred mg. of crude polysaccharide powder was wetted with ethanol and dissolved in 0.1 N-NaOH by heating in a boiling water bath for several minutes. The resulting suspension was neutralized to the phenolphthalein end point and to 50 ml. of liquor was added 2 g. hydrated sodium acetate, 10 ml. chloroform and 1 ml. *n*-butanol. After shaking for 10 min. in a separating funnel, the aqueous layer was removed and dialysed against running distilled water for 3 days. The non-diffusing polysaccharide was precipitated by 2 vol. cold ethanol, allowed to stand for 24 hr. at 2°, centrifuged and dried by grinding in ethanol and finally in ether. The resulting powder was dried *in vacuo* over P₂O₅. The purified polysaccharide (3.5 mg.) was hydrolysed in 0.5 ml. of 2N-H₂SO₄ in a sealed tube at 100° for 2 hr. and neutralized with BaCO₃. Constituent monosaccharides were detected by descending paper chromatography using: (a) *n*-butanol:pyridine:water (10:3:3); (b) ethyl acetate:pyridine:water (10:4:3); (c) *n*-butanol:glacial acetic acid:water (4:1:5). Aniline phosphate (0.05 M) was the developing agent in each instance. Reference carbohydrates were D (+) glucose, D (+) xylose, L (+) arabinose, D (+) galactose and L (+) rhamnose. 'Starch' content was assayed by estimating reducing sugar as glucose (Somogyi, 1945), blue value was determined by the method of Hobson & MacPherson (1952), ash by ignition, and total nitrogen by the micro-Kjeldahl method.

RESULTS

Characterization of the organism

Isolate 4-13 is a Gram-negative curved rod most closely allied to *Bacteroides*. Hereafter it will be referred to as *Bacteroides amylogenes* n.sp., whose characteristics are the following:

Bacteroides amylogenes n.sp. is a Gram-negative, non-motile, non-flagellated, asporogenous, non-capsulated, anaerobic, iodophilic, curved rod (2.5 μ . long by 0.5 μ . wide) from the rumen of a sheep (Pl. 1, fig. 1). It occurs singly and in short chains of organisms superficially resembling spirilla. The organisms are quite fragile and crush easily when pressed between glass slides. The organism is mesophilic and grows best at 39°, slowly at 32°, and not at all at 22° or 55°. Colonies on X agar are small (1-2 mm.), circular, raised, mucoid and pearl white. Twenty-four to forty-eight hr. colonies stain purple to blue black when covered with Lugol's iodine solution (Pl. 1, fig. 2) and organisms from these colonies are uniformly purple (Pl. 1, fig. 3). Rumen liquor or other complex nitrogenous nutrient, bicarbonate and a fermentable carbohydrate are needed for good *in vitro* growth. The organism ferments D (+) xylose, L (+) arabinose, L (+) rhamnose, L (-) fucose, D (+) glucose, D (+) mannose, D (+) galactose, D (-) fructose, glucosamine hydrochloride, sucrose, maltose, lactose, cellobiose, trehalose, melibiose, raffinose, salicin, aesculin and soluble pentosans with the production of acid and an intracellular iodophilic poly-

saccharide. It does not ferment L (–) xylose, D (+) lyxose, sorbose, adonitol, sorbitol, mannitol, dulcitol, inositol, α -methyl-glucoside, glycogen, amylose, soluble starch or cellulose. H_2S is produced from cysteine hydrochloride. Indole not formed. Catalase-negative.

Although *Bacteroides amylogenes* possesses a distinct cell wall when stained by Robinow's (1945) method, it appeared to bend and flex to and fro when observed in capillary tube preparations. This motion did not result in motility or physical progress as far as could be determined. The organism is obligately anaerobic and fails to grow in the absence of suitable reducing agents (as 0.1 % (w/v) cysteine hydrochloride), or when the resazurin indicator is pink to any degree. It survived 5 days of exposure to atmospheric oxygen, and subcultures so treated grew readily upon incubation under anaerobic conditions. Examination of stained preparations made from tubes in which the resazurin indicator showed a trace of oxygen present revealed long festoons and strands of organisms, many possessing 'bulbs' (Pl. 1, fig. 4) typical of those described by Hughes (1956). Probably these are pathological manifestations elicited by growth in the presence of inhibitory amounts of oxygen. Subculture and incubation of these forms under anaerobic conditions resulted in cells of 'regular' morphology.

Polysaccharide formation and utilization studies

The data in Table 1 reveal that a number of compounds, all carbohydrates, are transformed to iodophilic polysaccharide. It should be emphasized that in every case of growth iodophilic polysaccharide was produced. Notable among compounds not supporting growth were sugar alcohols, dicarboxylic acids, and hexosans except inulin, and to a lesser extent, dextrin. Soluble pentosans were readily fermented, whereas holotrich starch (Forsyth & Hirst, 1953) and purified *Bacteroides amylogenes* polysaccharide were not fermented. The pentoses are of some interest, particularly since C_5 units must be degraded and resynthesized to the C_6 units of the polysaccharide (see below). D (+) Lyxose, a pentose not found in nature, and L (–) xylose were not fermented; and D (–) arabinose and D (+) ribose supported only fair (1+) growth, yet these colonies were intensely iodophilic.

Bacteroides amylogenes grew poorly (2+ at best) on the first eight media listed under 'nutritional requirements'. Medium 9 supported growth equal to that obtained on X agar. It is significant that this organism did not grow well (2+) on X agar without bicarbonate (medium 7), and not at all on defined medium 8.

In Table 2 is shown the result of a typical experiment on the effect of various factors on the utilization of stored iodophilic polysaccharide by *Bacteroides amylogenes*. It was found to be readily utilized in 0.066 M-phosphate buffer (pH 7.0) under N_2 , and the addition of 1 % (w/v) D (+) xylose or D (+) glucose did not retard the process. In fact, after 5 hr. at least 95 % of the added carbohydrate remained unused. Utilization was virtually nil in 0.066 M-phosphate buffer at pH 5.0 under N_2 , or in 'tris' (tris-(hydroxymethyl)-aminomethane), pH 7.3, under N_2 , and it was noticeably retarded

in 0.04 M-carbonate + bicarbonate buffer (pH 7.0) in air. Addition of Casitone, acid hydrolysed casein, or strained rumen liquor was without effect, and there was no appreciable depletion of polysaccharide in the boiled cell preparation during 5 hr.

Table 2. *Utilization of iodophilic polysaccharide under various test conditions*

Each tube had a final volume of 3.0 ml., including cell suspension (0.28 mg. N/ml.) adjunct and buffer under appropriate O₂-free atmosphere at 39°. All zero hr. readings were o.d. = 1.00 at 640 mμ.

Buffer	pH	Atmo- sphere	Adjunct or treatment	EEL readings after (n) hr.		
				(1)	(3)	(5)
0.04 M-HCO ₃ ⁻	7.0	CO ₂	—	0.95	0.90	0.87
0.1 M 'tris'	7.3	N ₂	—	0.97	0.96	0.95
0.066 M-PO ₄	5.0	N ₂	—	0.97	0.97	0.97
0.066 M-PO ₄	7.0	N ₂	Cells boiled 3 min.	0.98	0.98	0.97
0.066 M-PO ₄	7.0	{ Air	—	0.98	0.94	0.92
		{ N ₂	0.3 % (w/v) Casitone	—	0.74	0.70
		{ N ₂	0.3 % (w/v) acid hydrolysed casein	—	0.72	0.71
		{ N ₂	0.3 % (w/v) strained rumen liquor	—	0.72	0.72
		{ N ₂	1.0 % (w/v) D (+) xylose	0.87	0.71	0.61
		{ N ₂	1.0 % (w/v) D (+) glucose	0.87	0.68	0.68
		{ N ₂	—	0.86	0.77	0.67

Xylose fermentation and polysaccharide identification

In Table 3 are shown the products of D (+) xylose fermentation by *Bacteroides amylogenes*. These results indicate that 1.7 % of the carbon fermented can be accounted for in lactic acid, and 89 % in the volatile fatty acid fraction

Table 3. *Products of xylose fermentation by Bacteroides amylogenes*

	Acetic	Propionic	Butyric	Lactic
mmole/l.	18.6	5.3	38.6	1.3
% C of xylose used (46.3 mmole/l.)	16.1	6.9	66.7	1.7
	91.4 %			

the chief constituent of which is butyric acid. A typical analysis of the intra-cellular polysaccharide on a dry weight basis was: $[\alpha]_D^{25} = +178^\circ$ ($c = 0.432$ in 50 % (w/v) CaCl₂ solution); 'starch' content, 89 % after acid hydrolysis; blue value 11.6 colorimeter units; total nitrogen, 0.1 %; ash nil. The only sugar found in the acid hydrolysate by paper chromatography was glucose. At pH 6 and 37° the extracted polysaccharide was readily hydrolysed by the freeze-dried bacterial α-amylase prepared from a rumen streptococcus by Hobson & MacPherson (1952).

DISCUSSION

Species of the genus *Bacteroides* are not uncommonly found associated with the intestinal tract of mammals. Hungate (1950) and Bryant & Burkey (1953) described *B. succinogenes* as an important cellulolytic organism of the bovine rumen. In so doing, the characterization of the genus *Bacteroides*, hitherto described mainly in terms of pathological processes and medical diagnostic bacteriology (*Bergey's Manual*, 1948), was extended to include cellulolysis and the production of characteristic end products therefrom. Additional work by Bryant & Doetsch (1954*a, b*) established some of the nutritional requirements of *B. succinogenes*. The organism studied in the present work has properties which most closely ally it to the rumen *Bacteroides* spp. group, i.e. it is a Gram-negative, non-flagellated, asporogenous, curved rod with rounded ends. Although it cannot hydrolyse cellulose and is iodophilic, it does attack soluble pentosans (hemicelluloses), requires bicarbonate, factors in rumen liquor and a fermentable carbohydrate for *in vitro* proliferation. It produces end products typical of those found in normal rumen liquor from a variety of carbohydrates. Bryant & Burkey (1953) isolated many strains of bacteria from the bovine rumen which were similar to the 'less actively cellulolytic rod' of Hungate (1950). Many of their strains failed to attack cellulose even after prolonged incubation. Huhtanen & Gall (1953) also isolated Gram-negative curved rods similar to those of Hungate. It is interesting to note that some of Hungate's cellulolytic isolates were considered to migrate through agar in a manner similar to the cytophagas but he could not observe any creeping or bending movement. *B. amylogenes* is clearly flexible when examined in capillary tube preparations or when the phase microscope is used.

Despite similarities and differences of the rumen *Bacteroides* spp. group previously described and *B. amylogenes*, it is emphasized that the taxonomic assignment is purely provisional. It is possible that another, more suitable, place may be found. It might be contended that *B. amylogenes* be considered with the genus *Desulphovibrio*. Lewis (1954) found that sulphate reduction in the rumen occurs rapidly and suggested that species of this genus might be involved. However, absence of flagella and apparent failure to reduce inorganic sulphate to H_2S argue against this consideration. Great difficulty is encountered in attempting to classify bacteria from unique environments (as the rumen), using a system wherein generic descriptions are based mainly on medically important members. Yet, it is probably better to assign names to isolates, however tentative, rather than apply taxonomically meaningless letters or numbers to them.

The bicarbonate requirement of *Bacteroides amylogenes* is consistent with observations of Hungate (1950) and Bryant & Burkey (1953). Although the nutritional requirements of *B. amylogenes* were not studied systematically it is probable that some of the unknown factors supplied by rumen liquor are to be discovered among the constituents of medium 8. Yeast extract and proteose-peptone alone, or the completely defined medium alone, do not support good growth, whereas they do so when in combination. X agar, a medium in which

the only non-defined constituent is rumen liquor, likewise supports luxuriant growth. Obviously the factors required are in rumen liquor, an observation made by Hungate (1950) and Bryant & Doetsch (1954*a*). In general, it appears that commercially available peptones, usually adequate for cultivation of nutritionally fastidious bacteria, do not contain all the growth factors required for adequate *in vitro* growth of many authentic rumen bacteria. McNeill *et al.* (1954) came to this conclusion after a study of the nutritional requirement of the total rumen bacterial population. Wilson & Briggs (1955) have been unable to confirm this and found 'reinforced clostridial medium' of Hirsch & Grinsted (1954) a satisfactory replacement for rumen liquor.

The foregoing studies suggest that certain purely physiological criteria might be advanced for judging the authenticity of a bacterium isolated from the rumen. These would include: (1) optimum temperature about 39°; (2) ability to grow anaerobically and consequently a need for some reducing agent in media devised for *in vitro* cultivation; (3) requirement for some organic factor for *in vitro* growth, the factor to be found in rumen liquor and not necessarily in commercially available peptones; (4) production of fatty acids from amino acids and carbohydrates, these to be characteristic of those found in normal rumen liquor. Undoubtedly other conditions can be added as work progresses.

Bacteroides amylogenes survived 5 days in the presence of atmospheric O₂. It did not grow under these conditions, but did so promptly when subcultured and incubated anaerobically. There are reports in the literature stressing elaborate precautions taken to exclude the atmosphere from rumen liquor samples. It would appear that they are largely unnecessary. Oxygen may be inhibitory to authentic rumen bacteria; it is very doubtful whether it is lethal (Doetsch *et al.* 1952). The transfer of the rumen bacterial flora from mature animal to young must of necessity proceed through space containing oxygen, and it is likely that such organisms suspended in rumen liquor are adequately protected from any ill-effects of atmospheric O₂ by reducing substances contained therein.

Bacteroides amylogenes can transform into 'starch' a portion of any compound which serves as an energy source (Table 1). In this regard it is unlike iodophilic strains of *Streptococcus bovis* (Hobson & Mann, 1955) which produce iodophilic polysaccharide only from a very limited range of carbohydrates. Iodophilic polysaccharide also is produced from many carbohydrates by mixed suspensions of bovine rumen bacteria (Robinson *et al.* 1955). The transformation of energy sources to polysaccharide is an important but not exclusive characteristic of many authentic rumen bacteria. The reaction may represent a mechanism, developed during the evolution of the rumen bacterial flora, for dealing with an excess of soluble fodder carbohydrate.

The 'starch' is a reserve substance which is used during starvation periods. The utilization of this internal polysaccharide (probably via a phosphorylase, but not excluding a non-diffusible amylase) proceeds even in the presence of exogenous soluble carbohydrates. Either additional factors are required or conditions under which the experiments are performed prevent the utilization

of them. In any event, mixed suspensions of rumen bacteria readily utilize exogenous soluble carbohydrate and 'spare' their internal polysaccharide, or add to it (Gibbons *et al.* 1955) when continuous growth is not possible. *Bacteroides amylogenes*, in resting suspensions, does not produce polysaccharide even when its internal supply is depleted. Depletion requires 3-5 hr. at 39° in a resting suspension, a period of time which perhaps leaves the organisms moribund.

The reserve polysaccharide of *Bacteroides amylogenes* is typical of those so far described for authentic rumen organisms, i.e. it is composed solely of glucose units. It may be emphasized that all known intracellular, as distinct from capsular polysaccharides, are glucose polymers (Oxford, 1951; Gibbons *et al.* 1955; Hobson & Mann, 1955). The good yield of polysaccharide in this instance was due to the relatively large quantities of 'starch' stored as well as the ease with which the organisms can be ruptured.

In line with the above criteria for establishing the authenticity of a bacterium isolated from the rumen, it is of interest to note that the end products from D (+) xylose fermentation are typical of compounds found in rumen liquor. The negligible lactic acid production by *Bacteroides amylogenes* was not unexpected since other pentose-fermenting rumen bacteria do not produce this metabolite from pentoses (Heald, 1952; Doetsch *et al.* 1953). It should be borne in mind, that organisms other than the lactic acid bacteria may be responsible for the occasional traces of lactic acid found in normal rumen liquor, or the large amounts found in abnormal rumen liquor.

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EXPLANATION OF PLATE

- Fig. 1. Crystal violet stain of 20 hr. culture grown on X agar. $\times 880$.
- Fig. 2. Colonies of 20 hr. culture grown on X agar and covered with Lugol's iodine solution. $\times 2$.
- Fig. 3. Wet preparation of Lugol's iodine-treated cells taken from 20 hr. culture grown on X agar. $\times 880$.
- Fig. 4. Long forms and 'bulbs' produced in presence of toxic amounts of oxygen. $\times 850$.

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R. N. DOETSCH, B. H. HOWARD, S. O. MANN AND A. E. OXFORD—POLYSACCHARIDE
PRODUCTION BY A RUMEN BACTERIUM. PLATE 1

(Facing p. 168)