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The Occurrence and Identification of Intracellular Polyglucose Storage Granules in *Methylococcus* NCIB 11083 Grown in Chemostat Culture on Methane

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Abstract. The accumulation of intracellular storage granules (0.03 – 0.5 μm) by *Methylococcus* NCIB 11083 when grown under conditions of ammonia limitation with methane as the sole source of carbon and energy was inversely proportional to the dilution rate. The isolated material was composed entirely of glucose residues and the infra-red spectrum exhibited characteristic absorption bands at 925 cm^{-1}, 845 cm^{-1} and 745 ± 4 cm^{-1}, indicating the presence of α (1 → 4) glycosidic linkages. The polymer dissolved in hot water to give an opalescent solution that formed a violet iodine complex with an absorption maximum at 550 nm, identical to that observed with reference amylopectin. The percentage of the polysaccharide released as maltose by the action of β- and α-amylases was 55 – 64% and 80 – 90% respectively, values very similar to those obtained by the action of these enzymes on reference amylopectin and glycogen. Methylation analysis indicated that the average interior and exterior chain lengths of the polymer were 2.7 and 10.0 glucose units respectively and confirmed that the *Methylococcus* polyglucose is a branched polymer composed of units joined by 1 → 4 and 1 → 6 linkages. The number average molecular weight of the polymer is $2-4.5 \times 10^5$. The stored polymer was metabolised by the organism and its metabolism resulted in the synthesis of protein.

Key words: *Methylococcus* – Methane – Chemostat – Ammonia limitation – Intracellular polyglucose.

A wide range of bacterial species is capable of storing glycogen or starch-like polymers intracellularly (Eidels et al., 1970; Strasdine, 1968; Tosic and Walker, 1950). *Methylococcus* NCIB 11083 belongs to the type 1 group of methane utilising organisms and has the ribulose monophosphate cycle of formaldehyde fixation. This paper reports the intracellular accumulation of a polymer of the glycogen/amylopectin type in *Methylococcus* NCIB 11083 grown in mineral salts medium with methane as the sole source of carbon and energy. The role of the intracellular polymer in terms of an energy reserve function is discussed.

Abbreviations. GLC = gas liquid chromatography; MS = mass spectroscopy; PAAN = peracetylated aldononitriles

Materials and Methods

Organism

A pure culture of *Methylococcus* NCIB 11083 and a mixed culture of *Methylococcus* NCIB 11083 plus four heterotrophic bacteria were employed as described previously (Linton and Buckee, 1977).

Growth Conditions

The medium used and the growth conditions employed in chemostat culture have been described previously (Linton and Buckee, 1977). Purity of *Methylococcus* in chemostat culture was established by daily checks involving inoculation of 100 ml shake flasks containing 25 ml sterile nutrient broth (Oxoid, CM_1) Lab Lemco broth (Oxoid, CM_{15}) and potato dextrose broth with 1 ml of culture from the chemostat. The flasks containing each type of medium were incubated at 25° C, 37° C and 42° C for 5 days. Growth in any of the flasks was indicative of contamination. Viable counts of *Methylococcus* NCIB 11083 were carried out on mineral salts medium (Linton and Buckee, 1977) containing 1.5% 'Lab m' agar (London Analytical and Bacteriological Media Ltd., London). Incubation was at 42° C, with methane as the sole source of carbon and energy. It is difficult to obtain values for the absolute viability of *Methylococcus*, nevertheless, this method is sensitive enough to detect relative changes in viability.

Degradation of Intracellular Polyglucose by Resting Cell Suspensions

Pure cultures of *Methylococcus* NCIB 11083 containing approximately 6% or 19% of the dry weight as polyglucose were harvested from chemostat culture, centrifuged and resuspended in mineral salts medium and incubated at 42° C on a rotary shaker in the absence of added carbon substrate and the disappearance of polymer and

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culture viability assayed over 150 h. In similar studies washed cells were incubated in mineral salts medium containing $Na_2\ ^{35}SO_4$ and the incorporation of radiolabel into cellular protein, concomitant with polyglucose metabolism, was followed. In a typical experiment, 0.4 g dry wt of cells were incubated with shaking at 30° C for 90 h in medium supplemented with 4.03 μ Ci of $Na_2\ ^{35}SO_4$. Samples of suspension were taken for polyglucose analysis at 0 and 90 h. After incubation the cells were washed and fractionated by successive treatments with ethanol and hot trichloroacetic acid to obtain a 'protein fraction' as described by Cripps (1973). The aqueous suspension of protein was radiocounted in PCS scintillator (Amersham/Searle Corp. Illinois, U.S.A.).

Isolation of Polysaccharide

Isolation of intracellular polysaccharide was based on the method of Zevenhuizen (1966). An equal volume of KOH (20 %) was added to a cell suspension (50 mg ml^{-1}) and the mixture was heated to 100° C for 1 h, allowed to cool and centrifuged. Ethanol (two volumes) was added to the supernatant and the precipitated polysaccharide was collected by centrifugation, redissolved in hot water, acidified to pH 5 with 2 N-HCl and recentrifuged to remove insoluble material. Ethanol was then added to the opalescent supernatant solution to a final concentration of 45 %. The precipitated polysaccharide was redissolved in hot distilled water and reprecipitated with ethanol. This procedure was repeated several times until no distinguishable absorbance maxima were observed in the range 240 – 280 nm. The aqueous solution of the polysaccharide was then successively dialysed against running tap water and distilled water for 24 h and finally precipitated with ethanol and sequentially washed twice in ethanol and diethylether, rotary evaporated at 35° C and then dried under vacuum at 50° C.

Analyses

Isolated polysaccharide (2 mg) was heated to 95° C for 16 h with 2 ml of 0.24 M-H_2SO_4 in a sealed vial. The acidic solution was neutralised with Dowex AG2-X8 (HCO_3^-) anion-exchange resin, filtered through glass wool to remove the resin and the volume made to 10 ml with distilled water. The solution was then assayed for glucose using the glucose oxidase system (Boehringer, Mannheim). The quantity of stored polymer in whole freeze dried bacteria was similarly assayed.

Analysis of bacteria and polysaccharide carbon, nitrogen, hydrogen and phosphorus content and bacterial amino acids was conducted as described previously (Linton and Buckee, 1977). Poly-β-hydroxybutyrate was assayed by the method of Law and Slepecky (1961).

Iodine-Polysaccharide Complexes

The absorbance of the *Methylococcus* polysaccharide-iodine complex [(Archibald et al., 1961) (approximately 0.25 mg polysaccharide in 1 ml iodine reagent)] was recorded in the region 400 – 700 nm and compared with those of the iodine complexes of glycogen (rabbit, Sigma type III), amylose (potato, Sigma type III) and amylopectin (potato, Koch-Light). The iodine reagent was prepared by adding 20 ml of I_2 + KI (0.2 % and 0.4 % respectively) to 100 ml of 0.1 M citrate buffer, pH 6.0, and diluting the mixture with 170 ml of distilled water.

Infra-Red Analysis

The infra-red spectrum of isolated intracellular polysaccharide of *Methylococcus* NCIB 11083 was recorded using a Perkin-Elmer 237 spectrophotometer and compared with that of glycogen, amylose, starch (Sigma) and amylopectin using the KBr-disc technique (Barker et al., 1956).

Determination of Chain-Lengths

Chain-length parameters of polysaccharides, before and after exhaustive β-amylolysis, were determined by methylation analysis using the Hakomori (1964) procedure. Polysaccharide samples (20 – 30 mg) were dried in vacuo at 50° C and dissolved in 2 ml of dry dimethyl sulfoxide under dry N_2. Dimethylsulfinyl sodium reagent (1 ml; ~2 M) was added and the mixture was incubated at ambient temperature for 7 h with frequent agitation. Methyl iodide (1 ml) was then added dropwise with cooling. After 45 min, the mixture was added to 10 ml of water and extracted with 3 × 10 ml of $CHCl_3$. The $CHCl_3$ solutions were dried with Na_2SO_4 and the permethylated polysaccharides were recovered by removal of solvent by evaporation.

Depolymerisation was achieved by a brief formolysis procedure (2 ml of 90 % HCOOH at 100° C for 1.5 h) followed by hydrolysis with dilute H_2SO_4 (0.25 M, 100° C, 18 h). Sulphate ion was removed from hydrolysates with Dowex AG2-X8 (HCO_3^-) anion-exchange resin which was washed successively with water and methanol. Combined washings were concentrated to dryness at 40° C.

Partially methylated glucose derivatives were converted into their peracetylated aldononitriles (PAAN) by successive treatment in sealed tubes with i) 20 mg of hydroxylamine hydrochloride in 0.4 ml of dry pyridine at 100° C for 40 min and ii) 0.5 ml of acetic anhydride at 100° C for 10 min. Solvents were removed in a stream of N_2 at 40° C and the residue was dissolved in 0.2 ml of $CHCl_3$ prior to analysis by GLC-MS.

GLC was performed at 195° C on a column (175 cm × 3 mm) of 3 % neopentyl glycol succinate on Chromosorb W-AW using N_2 as a carrier at 60 ml/min. Peak areas were computed by a Hewlett Packard 3380 integrator. The detector response was considered to be equal for each PAAN derivative and the relative proportions were taken to reflect the ratios of the underivatised methyl sugars in the hydrolysate. For GLC-MS analysis, the GLC conditions were similar except He was used as the carrier at 40 ml/min and the gas-chromatograph was coupled to a MS-30 mass-spectrometer with a VG Data System 200 computer attached.

The identity of the PAAN derivatives was established by comparison of their retention times with those of known compounds and confirmed by their characteristic mass spectra. Average chain length ($\overline{CL}$), average external chain length ($E\overline{CL}$) and average external chain length ($I\overline{CL}$) of the polysaccharide were as defined by Kindt and Conrad (1967) and were calculated from the relative proportions of the methyl ethers of glucose formed by GLC in hydrolysates of permethylated parent polysaccharide and β limit dextrins.

Conditions of Amylolysis

β-Amylolysis. Polysaccharide (5 mg) was dissolved in 1 ml of distilled water and 2.0 ml of acetate buffer (0.2 M, pH 4.6) and 0.1 ml of β-amylase (Sigma type IB, 25 mg protein ml^{-1}) were added. The solution was made up to 5 ml and incubated at 30° C for 24 h. When required, the β-limit dextrin was recovered by ethanol precipitation as above.

α-Amylolysis. Polysaccharide (5 mg) in 1 ml of distilled water was mixed with 0.65 ml of 0.5 % NaCl, 0.65 ml of 0.1 M-citrate buffer (pH 6.2) and 0.1 ml of α-amylase (Sigma type IA, 25 mg protein ml^{-1}). The volume was made up to 5 ml and the solution was incubated at 30° C for 24 h.

Both the α- and β-amylase contained measurable maltase activity. An approximate correction for this activity was obtained from

parallel incubation containing only maltose (5 mg). Determination of reducing sugars was by the method of Somogyi (1952) and the results expressed as the percentage of the polymer converted to maltose reducing equivalents.

Molecular Weight Estimation

The proportion of reducing end-groups in the *Methylococcus* polyglucose molecule and other reference polysaccharides were estimated using the Somogyi (1952) method. Polymer solution (2 ml, containing 5–16 mg ml^{-1} depending on solubility) was mixed with 2 ml of Somogyi copper reagent and heated in a boiling water bath for 45 min. The mixture was cooled and 2 ml of the arsenomolybdate reagent was added, mixed thoroughly and the absorbance at 520 nm was recorded. Maltose was used as a standard. The molecular weight was calculated from the determined μmoles of reducing sugar per g of total carbohydrate assuming one reducing group per molecule.

Electron-Microcopic Examination

An aqueous suspension of bacteria was fixed in 3% glutaraldehyde in 0.1 M Sørenson's phosphate buffer pH 7.4 (Sabatini et al., 1963) for 2 h. The sample was then postfixed in 1% osmium tetroxide in 0.1 M Sørenson's phosphate buffer (Sabatini et al., 1963) and dehydrated through a series of ethanol concentrations to absolute ethanol and embedded in standard Spurr resin (Spurr, 1969). Thin sections were cut using a LKB Ultratome III. The sections were stained either with (a) saturated uranyl acetate in 50% ethanol for 30 min, washed with 50% ethanol and distilled water and then double stained with alkaline lead citrate (Reynolds, 1963) for 30 min, washed with 0.02 N NaOH and distilled water; or with (b) a specific stain for polysaccharide (Flood, 1970).

Measurement of K_m Values

The effect of various concentrations of substrate on the respiration rate of samples taken from a chemostat containing a pure culture of *Methylococcus* NCIB 11083 was detected using an oxygen electrode cell as described previously (Linton et al., 1975).

Results

Characteristics of the Intracellular Polyglucose Granules of Methylococcus NCIB 11083

Hydrolysis of the purified intracellular polyglucose from *Methylococcus* NCIB 11083 yielded 100% glucose. The carbon and hydrogen contents of *Methylococcus* polyglucose (C = 42.6%, H = 7.1%, N and $P < 1\%$) were similar to those of reference glycogen and amylopectin [glycogen, (rabbit liver) C = 40.4%, H = 6.1%, N and $P < 1\%$; amylopectin, (potato) C = 40.5%, H = 6.4%, N and $P < 1\%$]. The nitrogen and phosphorus content in all samples examined were below the limit of detection of the assay used.

The infra-red spectrum of the polysaccharide was almost identical to that observed with the reference polysaccharides, glycogen, amylose, starch and amylopectin. Absorbance peaks in the region 960–720 cm^{-1} characteristic of D-glucopyranose residues (Barker et al., 1956) were observed. Polymers with α-glucosidic linkages absorb at 850 cm^{-1} (types 1 and 3 absorption) and this peak does not occur in the spectrum of polysaccharides containing β-linkages. In addition, α-polyglucosans of the starch type (α-[1→4] glucosidic linkages) absorb at 934 ± 4 and 758 ± 2 cm^{-1} (types 1 and 3 absorption respectively). These absorbance peaks were present in the *Methylococcus* intracellular polysaccharide and in the reference polysaccharides (Fig. 1).

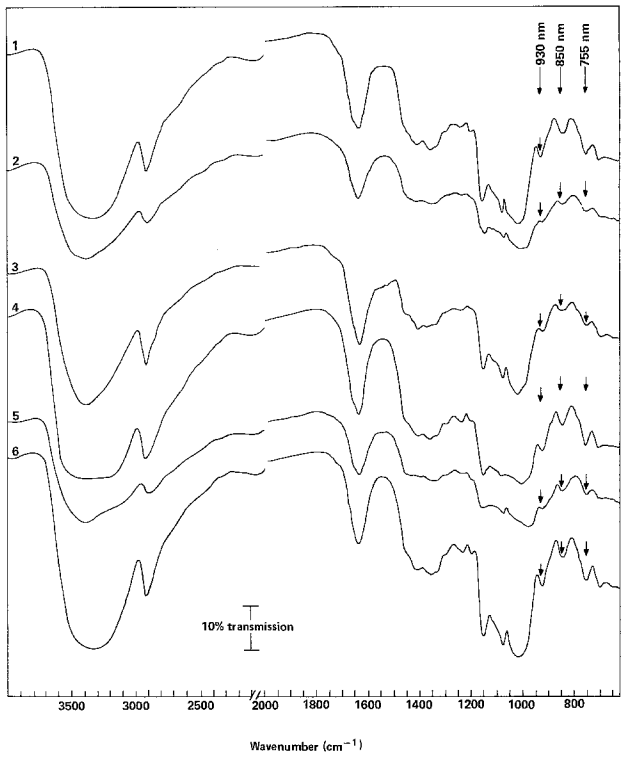

Fig. 1. A comparison of the infra-red spectra of polyglucose isolated from *Methylococcus* sp. with reference to polysaccharides of the starch-glycogen type. *1* glycogen (oyster); *2* glycogen (rabbit liver); *3* *Methylococcus* sp. polyglucose; *4* amylopectin (potato); *5* starch (potato); *6* amylose (potato)

The spectra of various known polysaccharide-iodine complexes were compared with that of the polyglucose isolated from *Methylococcus* NCIB 11083 (Fig. 2). The iodine-complex of a strongly branched polysaccharide has an absorbance maximum at a shorter wavelength than that of an unbranched molecule (Archibald et al., 1961). Thus the absorbance maximum of glycogen, a highly branched glucose polymer was 495 nm whereas that for the unbranched polymer, amylose, was 630 nm (Fig. 2). The absorbance maximum of the polyglucose isolated from

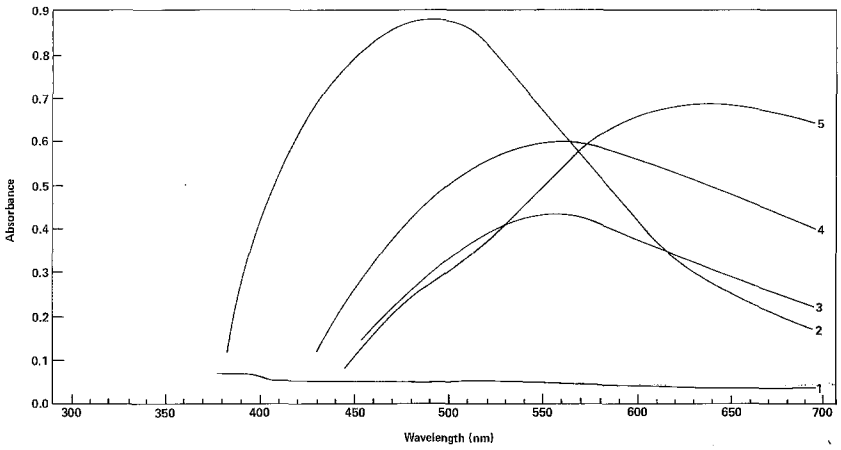

Fig. 2
Absorbance spectra of polysaccharide-iodine complexes. *1* iodine/iodine blank; *2* glycogen (rabbit, 0.14mg ml^{-1}); *3* amylopectin (potato, 0.10 mg ml^{-1}; *4* polyglucose isolated from *Methylococcus* sp. (0.25 mg ml^{-1}); *5* amylose (potato, 0.25 mg ml^{-1})

Methylococcus NCIB 11083 was 550 nm identical to that observed with the reference amylopectin (potato) (Fig. 2).

The action of α- and β-amylase on polyglucose isolated from *Methylococcus* NCIB 11083 was compared with the action of these enzymes on reference polysaccharides glycogen, starch, amylose and amylopectin. The percentage of the polysaccharide released as maltose reducing equivalents by the action of these enzymes [the α- and β-amylolysis limit (%)] is shown in Table 1. *Methylococcus* intracellular polysaccharide was attacked to the same extent as the reference glycogen and amylopectin by these two enzymes.

Methylation analysis of glycogen, amylopectin and *Methylococcus* polyglucose revealed, in each case, the same three partially methylated sugars (2,3,4,6-tetramethyl glucose, 2,3,6-trimethyl glucose and 2,3-dimethyl glucose), confirming that the *Methylococcus* polysaccharide was a 1→4 linked glucose polymer with 1→6 branch points. The efficiency of methylation was confirmed by the occurrence of equal amounts of the tetra- and di-methyl derivatives.

The chain length parameters, calculated from the methylation data, are shown in Table 2. These data indicate a distinct similarity between *Methylococcus* polyglucose and glycogen.

Determination of the number of reducing equivalents per g total carbohydrate permitted a crude estimation of the number average molecular weight of *Methylococcus* polyglucose and reference polysaccharides. Values of $2.2-2.5\times10^6$, $1.4-1.6\times10^5$, 1.1×10^6 and $2.3-4.5\times10^5$ were obtained for glycogen, amylose, amylopectin and *Methylococcus* polyglucose respectively.

Poly-β-hydroxybutyrate was not detected in bacteria harvested from chemostat culture either under ammonia or methane limitation.

Table 1. The percentage of polysaccharide released as maltose by the action of α- and β-amylase (amylosis limit, %) on polyglucose isolated from *Methylococcus* sp. and reference glycogen, starch, amylose and amylopectin

Polysaccharide	β-amylolysis limit (%)	α-amylolysis limit (%)
Glycogen (oyster)	58	86
Starch	57	–
Amylose	107	93
Amylopectin	48	85–95
Methylococcus polyglucose	55–64	80–90

Table 2. Chain lengths of amylopectin, glycogen and *Methylococcus* polyglucose derived from methylation-analysis and published data

Polysaccharide	Average chain length ($\overline{CL}$)	Average exterior chain length ($E\overline{CL}$)	Average interior chain length ($I\overline{CL}$)
Glycogen (Sigma)	13.2	9.3	2.9
β-Limit glycogen	6.4	(2.5)	
Amylopectin (Koch-Light)	20.3	15.2	4.1
β-Limit amylopectin	7.6	(2.5)	
Methylococcus polyglucose	13.7	10.0	2.7
β-Limit polyglucose	6.2	(2.5)	
Amylopectin (Archibald et al., 1961)	23	14–15	7–8
Glycogen (Archibald et al., 1961)	14	9–10	3–4

Growth Physiology and Polymer Formation

Mixed Culture under Ammonia Limitation. In the mixed culture, *Methylococcus* sp NCIB 11083 accounted for at least 85% of the bacterial population by number and

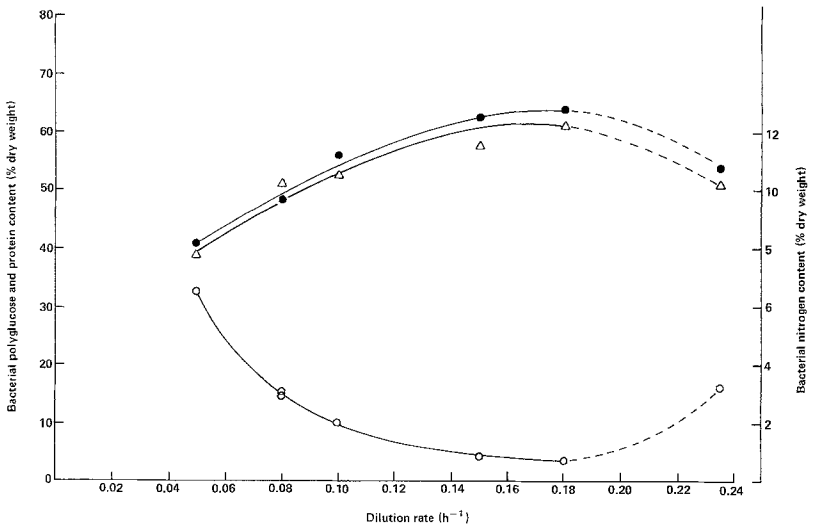

Fig. 3
The content of intracellular polyglucose, protein and nitrogen of a mixed culture containing *Methylococcus* NCIB 11083, as a function of the dilution rate under ammonia limitation. (△——△) bacterialnitrogen; (○——○) intracellular polyglucose; (●——●) bacterial protein (summation of amino acid content g per 100 g bacterial dry weight)

not less than 98 % of the bacterial biomass. Intracellular polyglucose granules were stored maximally at low dilution rates, 32.5 % of the bacterial dry weight were accounted for as glucose at a dilution rate of $0.05\,h^{-1}$. The polyglucose content of the culture under ammonia limitation was inversely related to dilution rate (Fig. 3). At low growth rates the bacterial nitrogen and protein contents were depressed and increased with increasing growth rate (decreasing intracellular polyglucose) (Fig. 3). However, during the washout phase when the culture was growing at its maximum growth rate, the bacterial polysaccharide content increased to 16 % of the dry weight and there was a decrease in the bacterial nitrogen and protein content (Fig. 3). Under these conditions the culture supernatant contained $0.2\,gl^{-1}$ ammonia and growth was probably limited by the Ca^{2+} content of the medium. An inverse relationship between intracellular polyglucose and bacterial nitrogen content was evident in bacteria grown under various growth conditions (Fig. 4). There was a strong correlation between the amount of intracellular polyglucose and the content of granules as seen by electron-microscopy (Fig. 5). The granules were electron transparent when stained with uranyl acetate/lead citrate but electron dense after treatment with iodine vapour.

Pure Culture of Methylococcus NCIB 11083. A pure culture of *Methylococcus* NCIB 11083 was grown under ammonia limitation. The major disadvantage of running a pure culture of this organism compared to a mixed culture is culture foaming caused by release of lysis products of *Methylococcus* into the culture supernatant (Linton and Buckee, 1977). Foaming causes changes in the culture volume and consequent alteration of dilution rate. Thus the culture takes considerable time to reach a steady-state, and initially exhibits

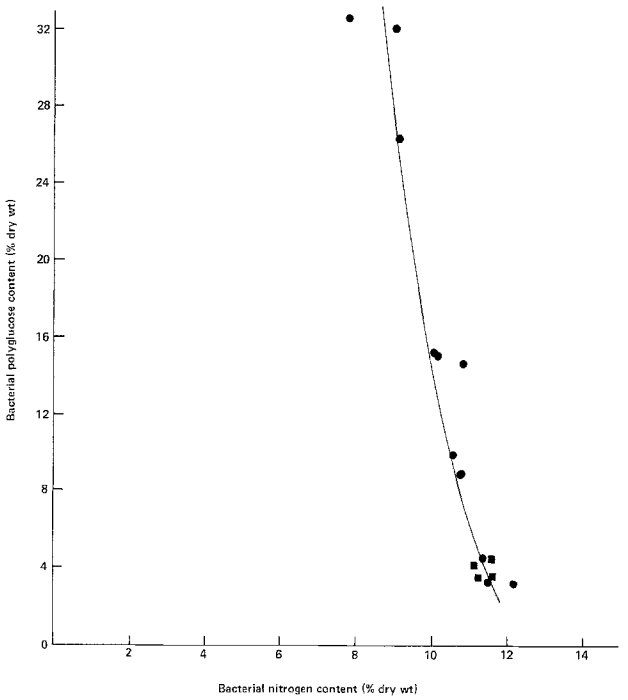

Fig. 4. The relationship between bacterial nitrogen and intracellular polyglucose content. (●——●) ammonia limitation at various dilution rates; (■——■) methane limitation; $D = 0.22\,h^{-1}$

oscillations in biomass and other physiological parameters, including the content of intracellular polyglucose.

Effect of Stored Polyglucose on Bacterial Viability. The effect of intracellular stored polyglucose on the survival of a pure culture of *Methylococcus* NCIB 11083 incubated at 42° C in mineral salts medium in the absence of an added carbon source is shown in Figure 6. Intracellular polyglucose was readily metabolised (at

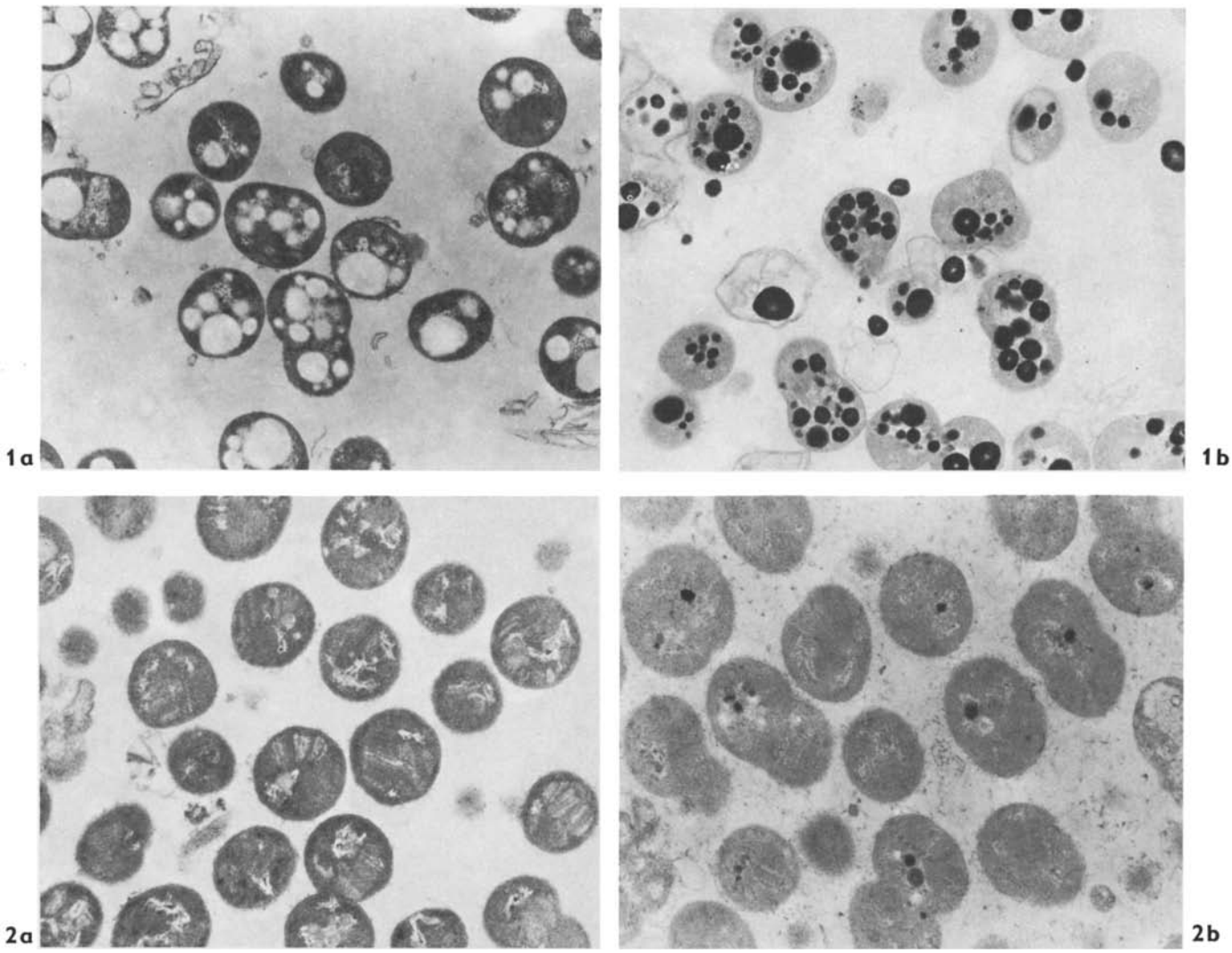

Fig. 5. Electronmicrographs of thin sections of *Methylococcus* sp. NCIB 11083, showing intracellular polyglucose storage granules. Bacteria grown under ammonia limitation. *1* $D = 0.05\ h^{-1}$, *2* $D = 0.18\ h^{-1}$; *a* stained with uranyl acetate/lead citrate, *b* stained with I_2 vapour

42° C) being exhausted after 50 and 120 h in bacteria containing 6% and 19% of their dry weight as polyglucose respectively. When the incubation temperature was reduced to 30° C only 56% of the intracellular polyglucose was utilised after 96 h. There appeared to be no change in culture viability during the depletion of the intracellular polyglucose.

Utilisation of Intracellular Polyglucose for Protein Synthesis. The metabolism of polyglucose was accompanied by the uptake of sulphur-35 into cellular protein. In the experiment described in "Materials and Methods", 0.77 µg of sulphur was incorporated in the protein fraction per mg of polyglucose oxidised. Since, under the experimental conditions used, 0.5% by weight of cellular protein is sulphur, and 60% of the biomass is protein, the sulphur uptake figure is equivalent to a yield of 0.26 g cells per g polyglucose metabolised.

Discussion

Methylococcus NCIB 11083 intracellular polysaccharide is a branched polymer composed of glucose units joined by $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages and has a molecular weight of approximately $2-4.5\times10^5$. The polysaccharide-iodine complex exhibited an absorbance maximum similar to that reported for amylopectin (Archibald et al., 1961), however, the average chain length and the average exterior and interior chain lengths were closely similar to those obtained for glycogen (Archibald et al., 1961). Moreover, the polysaccharide was attacked to the same extent as glycogen

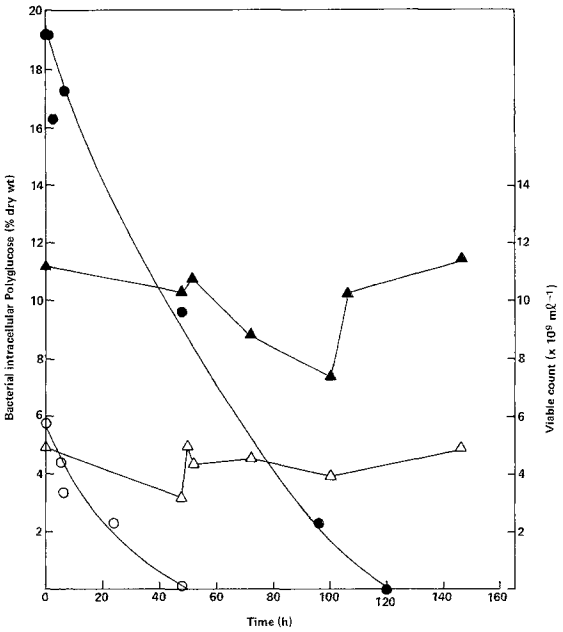

Fig. 6. The effect of intracellular polyglucose on the viability of *Methylococcus* NCIB 11083 incubated in carbon tree mineral salts medium at 42° C on a rotary shaker. Decrease in intracellular polyglucose content of bacteria containing (●——●) 19% and (○——○) 6% initial intracellular polyglucose. Viability of bacteria containing initial polyglucose contents of (▲——▲) 19% and (△——△) 6% respectively

by α- and β-amylases. Thus, the degree of branching closely resembled that of glycogen whereas the iodine-complex and molecular weight of the polymer indicated a similarity to amylopectin. This is the first report of the accumulation of intracellular glycogen/amylopectin in methane utilising bacteria, although the accumulation of poly-β-hydroxybutyrate in these organisms has been reported (Whittenbury et al., 1970). Indeed, Proctor et al. (1969) reported the presence of circular areas of low electron density in *Methylococcus capsulatus* which were not due to poly-β-hydroxybutyrate. These authors suggested that these structures were comprised of lipid. However, the accumulation of glycogen has been reported in the obligate methanol utiliser, *Methylobacillus glycogenes* (Yordy and Weaver, 1977).

The inverse relationship between intracellular polysaccharide accumulation and dilution rate under nitrogen limitation has been reported for other micro-organisms (Holme, 1952; Herbert, 1961) and the low values of bacterial nitrogen and protein at low dilution rates is a consequence of the accumulation of intracellular polysaccharide under these conditions.

At the critical dilution rate, growth was not limited by the nitrogen source but probably by the Ca^{2+} content of the medium. Under these conditions intracellular polyglucose accounted for 16% of the bacterial dry weight. Thus the accumulation of intracellular polysaccharide is not confined to conditions of low growth rate under nitrogen limitation but may occur when the organism is growing rapidly and growth is limited by the availability of some constituent of the growth medium other than the nitrogen source.

Methylococcus NCIB 11083 accumulated intracellular polyglucose when grown under ammonia limitation with the carbon source, methane, in excess and could utilise the accumulated polymer for protein synthesis in the absence of an exogenous carbon source. Thus, the criteria advanced by Wilkinson (1959) for the establishment of an energy-storage function for an intracellular polymer are met. As the intracellular polyglucose could be used to drive protein synthesis *Methylococcus* NCIB 11083 must be capable of generating energy and the carbon skeletons necessary for this process from the intracellular polyglucose. The rate of utilisation of the intracellular polyglucose was temperature dependent and although exhaustion of the resource occurred after 96 h at 42° C the rate of utilisation would be greatly reduced at the lower temperature prevailing in nature. Exhaustion of the intracellular polyglucose did not affect cell viability under laboratory conditions. However, the laboratory environment does not reflect conditions prevailing in nature and an answer as to whether the ability to store intracellular polyglucose by *Methylococcus* confers upon it a selective advantage over its competitors must await the development of techniques directly applicable to the measurement of growth and survival of these organisms in their natural environment.

Intracellular polyglucose polymers usually accumulate under conditions when the carbon and energy source is present in excess and growth is limited by some other essential nutrient such as nitrogen, phosphorus or sulphur (Dawes and Senior, 1973). In this carbon sufficient environment, the immediate selection pressure is for the efficient utilisation of the growth limiting nutrient. The utilisation of the carbon source may be regulated by modulation of the K_m for the substrate coupled to additional mechanisms regulating the rate of assimilation of the substrate as demonstrated for *Klebsiella aerogenes* growing on glycerol (Neijssel et al., 1975). However, if the organism cannot regulate the uptake of the substrate and over utilisation occurs, as found when *K. aerogenes* is grown on glucose (Neijssel and Tempest, 1975), the organism is faced with the problem of dealing with the excess carbon and reducing equivalents generated by this process. The affinity of *Methylococcus* NCIB 11083 for methane was high under carbon excess (ammonia limitation) or carbon limitation and it is suggested that the accumulation of intracellular polysaccharide is a symptom of the 'over utilisation' of methane.

Whether the 'over utilisation' of the carbon source leads to the excretion of 'overflow' products as de-

monstrated for *Klebsiella aerogenes* grown on glucose (Neijssel and Tempest, 1975) or is channelled into the synthesis of intracellular polysaccharide will depend on the evolutionary history of the particular organism being considered. That is, under carbon sufficient conditions the primary selection pressure will be the efficient utilisation of the growth limiting nutrient. Depending on the growth environment of a particular organism, secondary selection pressures will dictate whether the uptake of the carbon source is modulated, 'overflow' products excreted or the carbon conserved as intracellular polysaccharide. For example, an organism living in the gut of mammals may be supplied with its carbon source(s) fairly regularly and there may be no selective pressure to conserve the 'overflow' products whereas an organism in the soil is faced with periods of feast and famine and there may be a positive selective advantage in conserving the 'overflow' carbon compounds in the form of intracellular storage products.

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References

Archibald, A. R., Fleming, I. D., Liddle, M. A., Manners, G. A., Wright, A.: α-1,4-glucosans. Part XI. The absorption spectra of glycogen and amylopectin-iodine complexes. J. Chem. Soc. **1**, 1183–1190 (1961)

Barker, S. A., Bourne, E. J., Whiffen, D. H.: Use of infra-red analysis in the determination of carbohydrate structure. Meth. Biochem. Analys. **3**, 213 (1956)

Cripps, R. E.: Microbial metabolism of Thiophen-2-carboxylate. Biochem. J. **134**, 353–366 (1973)

Dawes, E. A., Senior, P. J.: The role and regulation of energy reserve polymers in micro-organisms. In: Adv. microb. physiol., Vol. 10 (A. H. Rose, D. W. Tempest, eds.), pp. 136–266. New York: Academic Press 1973

Eidels, L., Edelmann, P. L., Preiss, J.: Biosynthesis of bacterial glycogen. VIII. Activation and inhibition of the adinosine diphosphoglucose pyrophosphorylases of *Rhodopseudomonas capsulata* and of *Agrobacterium tumefaciens*. Arch. Biochem. Biophys. **140**, 60–74 (1970)

Flood, P. R.: Preliminary experiments with iodine in electron opaque stains for ultrathin sections. Proc. Soc. Franc. de Mic. Elec., pp. 431–432, Grenoble (1970)

Hakomori, S.: A rapid permethylation of glycolipid, and polysaccharide catalysed by methylsufinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) **55**, 205–208 (1964)

Herbert, D.: The chemical composition of micro-organisms as a function of their environment. In: Symp. Soc. Gen. Microbiol., Vol. 11, pp. 391–416 (G. G. Meynell, H. Gooder, eds.). Cambridge, England: University Press 1961

Holme, T.: Bacterial synthesis during limited growth. The formation of glycogen and of nitrogenous compounds in *Escherichia coli* B. as influenced by nutritional defficiences. Doctoral Thesis, Karolinksa Institute, Stockholm (1952)

Kindt, T. J., Conrad, H. E.: The role of primer in glycogen biosynthesis in *Aerobacter aerogenes*. Biochem. **6**, 3718–3729 (1967)

Law, J. H., Slepecky, R. A.: Assay of poly-β-hydroxybutyric acid. J. Bacteriol. **82**, 33–42 (1961)

Linton, J. D., Buckee, J. C.: Interactions in a methane-utilising mixed bacterial culture in a chemostat. J. Gen. Microbiol. **101**, 219–225 (1977)

Linton, J. D., Harrison, D. E. F., Bull, A. T.: Molar growth yields, respiration and cytochrome patterns of *Beneckea natriegens* when grown at different medium dissolved oxygen tensions. J. Gen. Microbiol. **90**, 237–246 (1975)

Neijssel, O. M., Hueting, S., Crabbendam, K. J., Tempest, D. W.: Dual pathways of glycerol assimilation in *Klebsiella aerogenes* NCIB 418. Arch. Microbiol. **104**, 83–87 (1975)

Neijssel, O. M., Tempest, D. W.: The regulation of carbohydrate metabolism in *Klebsiella aerogenes* NCIB 418 organisms growing in chemostat culture. Arch. Microbiol. **106**, 251–258 (1975)

Proctor, H. M., Norris, J. R., Ribbons, D. W.: Fine structure of methane-utilising bacteria. J. Appl. Bacteriol. **32**, 118–121 (1969)

Reynolds, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. **17**, 208–212 (1963)

Sabatini, D. D., Bensch, K., Barrnett, R. J.: Cytochemistry and electron microscopy: the preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell. Biol. **17**, 19–58 (1963)

Somogyi, M.: Notes on sugar determination. J. Biol. Chem. **195**, 19–23 (1952)

Spurr, A. R.: A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**, 31–43 (1969)

Strasdine, G. A.: Amylopectin accumulation in *Clostridium botulinum* type E. Canad. J. Microbiol. **14**, 1059–1062 (1968)

Tosic, J., Walker, T. K.: *Acetobacter acidum mucosum* Tosic and Walker, n. sp., an organism forming a starch-like polysaccharide. J. Gen. Microbiol. **4**, 192–196 (1950)

Whittenbury, R., Phillips, K. C., Wilkinson, J. F.: Enrichment isolation and some properties of methane-utilising bacterial. J. Gen. Microbiol. **61**, 205–218 (1970)

Wilkinson, J. F.: The problem of energy-storage compounds in bacteria. Exptl. Cell. Res. Suppl. **7**, 111–130 (1959)

Yordy, J. R., Weaver, T. L.: Methylobacillus: A new genus of obligately methylotrophic bacteria. Int. J. System. Bacteriol. **27**, 247–255 (1977)

Zevenhuizen, L. P. P. M.: Function, structure and metabolism of the intracellular polysaccharide of *Arthrobacter*. Doctoral thesis, Wageningen (1966)

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