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FUNCTION, STRUCTURE AND METABOLISM OF THE INTRACELLULAR POLYSACCHARIDE OF *ARTHROBACTER*

L. P. T. M. ZEVENHUIZEN

Laboratory of Microbiology,
Agricultural University, Wageningen, The Netherlands

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1. INTRODUCTION

Polysaccharides often occur as cellular components in microorganisms. STACEY and BARKER (1960) have given a review of the occurrence, the structure, the function and the metabolism of these compounds.

The polysaccharides of microorganisms can be divided into three groups according to their morphological localization, viz. a. extracellular polysaccharides, located outside the cell wall and frequently termed capsular polysaccharides, b. cell wall polysaccharides and c. somatic or intracellular polysaccharides, located inside the cytoplasmic membrane.

One of the most important functions of the capsular polysaccharides is the resistance to unfavourable external circumstances they confer on the organisms (WILKINSON, 1958). The polysaccharide capsule increases resistance to attack by leucocytes, amoebae and bacteriophages. Thus, non-encapsulated, avirulent pneumococci are readily phagocytized when added to a suspension of leucocytes in normal serum, whereas encapsulated, virulent organisms are resistant to phagocytosis and multiply rapidly. Most encapsulated strains of *Escherichia coli* are resistant to bacteriophages.

Bacteria growing in the soil or on vegetation are more resistant to alterations in the water content of the medium by utilization of the hygroscopic nature of the capsular polysaccharides, preventing a too rapid loss or gain of water which would cause cell death (WILKINSON, 1958).

Cell wall polysaccharides form part of the composition and structure of the cell wall. The latter is composed of high-molecular components, usually in the form of complexes of proteins, lipids and polysaccharides.

Many microorganisms contain polysaccharides of the glycogen-starch type within their cells; these carbohydrates are distributed through the cytoplasm and function as energy reserves and carbon sources, e.g. the starch-like polysaccharide of *Clostridium butyricum* and the glycogen of yeast. The intracellular polysaccharides of *Arthrobacter*, which are studied in the present investigation, also belong to the latter type.

At least twenty monosaccharides and derivatives have been recognized as constituents of bacterial polysaccharides. These include: D-glucose, D-mannose, D-galactose, D-glucuronic acid, D-mannuronic acid, D-galacturonic acid, D-xylose, L-arabinose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, L-fucose, D-arabinose, L-rhamnose, D-fructose.

Polysaccharides can be divided chemically into two groups: the homopolysaccharides which are built up from one component sugar and the heteropolysaccharides which are built up from two or more component sugars.

In the following a review is given of a number of homopolysaccharides from microorganisms. The choice is limited to those polysaccharides completely built up from D-glucose, the so called polyglucosans.

1.1. Intracellular polysaccharides of the starch-glycogen type in microorganisms

It is generally accepted that the reserve polysaccharides of this type, particularly glycogen, play an important role in most, if not all, microorganisms. However, these carbohydrates have been studied only in a relatively small number of microorganisms. In most cases the investigators have dealt with only one aspect of this subject, for instance the occurrence, the structure, the function or the metabolism of these polysaccharides.

The polysaccharides of the starch-glycogen type are built up from D-glucose residues, which are linked to each other by $\alpha(1\rightarrow4)$-glucosidic bonds. Straight chains of glucose residues then arise, as in amylose, built up from several hundreds of glucose residues per molecule. Branching may occur by $\alpha(1\rightarrow6)$-glucosidic linkages; a tree-like structure then arises, as in amylopectin and glycogen. Starch is a mixture of amylopectin and amylose. The average chain length ($\overline{CL}$) of these branched polysaccharides is defined as the number of glucose residues per mole non-reducing terminal glucose. This value is a measure of the degree of branching. For amylopectins $\overline{CL}$ = 20–25, for glycogens $\overline{CL}$ = 10–15, so that the latter group has a higher degree of branching (cf. Fig. 10).

Crystalline amylose has been obtained from cultures of a pathogenic yeast, *Torula histolytica* (Hehre, Carlson and Hamilton, 1949).

Starch has been isolated from *Corynebacterium diphtheriae*. It has been separated into two components, amylopectin and amylose, by way of the fractionation technique of Schoch. In this way amylose was obtained in crystalline form (Carlson and Hehre, 1949).

Clostridium butyricum forms a starch-like polysaccharide (Nasr and Baker, 1949).

From a strain of *Escherichia coli* Monod and Torriani (1948) obtained an extract, which upon incubation with maltose formed a polysaccharide. This product gave a blue colour with iodine (starch). In this reaction one molecule of glucose was liberated from each molecule of maltose utilized (see chapter 7). The reaction proceeded under the influence of amylomaltase. This enzyme was adaptive and was found only in bacteria which were cultivated on maltose.

The yeast species *Cryptococcus albidus* and *C. laurentii* form an extracellular polysaccharide, which shows resemblance with starch: $[\alpha]_D = +198°\cdot4$; $\overline{CL} = 44$. It consists of short chains of amylose (Kooiman, 1963).

Acetobacter acidum-mucosum forms a starch-like polysaccharide (Tosic and Walker, 1950).

The flagellate *Polytomella coeca* synthesizes and utilizes intracellular starch, although this organism is unable to utilize extracellular sugars as carbon sources. It contains a phosphorylase (Lwoff, Ionesco and Gutmann, 1950).

Neisseria perflava contains an amylosucrase, which transforms sucrose into an amylopectin-like polysaccharide (Hehre and Hamilton, 1946; 1948). Investigation of the enzyme system showed, that two enzymes are concerned.

Amylosucrase converts sucrose into an unbranched polysaccharide of the amylose type (see chapter 7). A second enzyme, analogous to Q-enzyme (cf. section 7.4.), converts amylose into a glycogen-like polysaccharide (HEHRE, HAMILTON and CARLSON, 1949).

The protozoon *Cycloposthium* forms a polyglucose which has an amylopectin-like structure. The mean chain length, determined by methylation and periodate oxidation, was found to be 23 (FORSYTH, HIRST and OXFORD, 1953).

Glycogen was isolated from *Saccharomyces cerevisiae*. End group assay by periodate oxidation indicated an average chain length of 12 glucose residues. When subjected to attack by β-amylase, the glycogen gave 50 per cent maltose (NORTHCOTE, 1953).

The ciliate *Tetrahymena pyriformis* forms a glucose polymer showing structural resemblance with animal glycogen. Mol. weight: 9.8×10^6; $[\alpha]^{18}_D=+195°$; $\overline{CL}=13$; β-amylase split off 44% as maltose (MANNERS and RYLEY, 1952).

The presence of glycogen was also demonstrated in *Mycobacterium*, viz. *M.phlei* (GERMAN, JONES and NADARAJAH, 1961) and in *M. tuberculosis* (CHARGAFF and MOORE, 1944; KENT and STACEY, 1949).

The glycogen isolated from *Bacillus megaterium* had the following properties: $[\alpha]^{20}_D=+173$; $\overline{CL}=9\pm1$ (periodate oxidation and methylation); β-amylase gave 43–46 per cent maltose. With iodine the glycogen gave a faint brown colour (BARRY, GAVARD, MILHAUD and AUBERT, 1952).

Escherichia coli contains glycogen (PALMSTIERNA, 1956). Glycogen has also been demonstrated in *Aerobacter aerogenes*. The isolated product gave a red-violet colour with iodine (STRANGE, DARK and NESS, 1961). MADSEN (1963) has studied the metabolism of glycogen in *Agrobacterium tumefaciens*.

1.2. OTHER POLYGLUCOSANS OF MICROORGANISMS

In this section some examples will be given of microbial polyglucosans, which have structures different from those of the polysaccharides of the starch-glycogen type. The functions of these polysaccharides are often also different.

From the cell wall of yeast a polysaccharide named glucan was isolated. Methylation studies of this yeast glucan showed that the glucose residues were mainly linked by β(1→3)-glucosidic bonds. This polysaccharide possessed a highly branched structure (BELL and NORTHCOTE, 1950).

Agrobacterium tumefaciens grown on sucrose, glucose or fructose as the sole carbon source, forms a water-soluble polyglucosan with low molecular weight. Investigation of the structure of this polysaccharide showed, that the glucose residues are linked by β(1→2)-glucosidic bonds (PUTMAN, POTTER, HODGSON and HASSID, 1950).

Acetobacter xylinum and *Acetobacter acetigenum* form cellulose, when the cells are growing in a carbohydrate-containing medium. Cellulose is a polyglucosan, in which the glucose residues occur in a straight chain linked by β(1→4)-glucosidic bonds. The enzymic synthesis of cellulose by a cell-free

particulate extract of *A.xylinum* has been studied by GLASER (1957). Incubation of the enzyme with uridine diphosphate glucose (UDPG) and soluble cellodextrins as receptors gave a water- and alkali-insoluble material, which was identified as cellulose. Both glucose-1-phosphate and glucose were inactive when incubated with the enzyme.

Leuconostoc mesenteroides and *Leuconostoc dextranicus* form extracellular dextran, when cultivated in a sucrose medium. The glucose residues in the main chains are linked by α(1→6)-glucosidic bonds. Branching takes place by α(1→4)-glucosidic and less frequently also by α(1→3)-glucosidic linkages. The enzymic synthesis of dextran is catalysed by dextransucrase (HEHRE, 1946; see also chapter 7).

Finally mention can be made of the luteose from *Penicillium luteum*, a polyglucosan with β(1→6) as the major glucosidic linkage (ANDERSON et al., 1939) and the nigeran from *Aspergillus niger* with long chains of more than 300 glucose residues, alternately linked by α(1→3) and α(1→4)-glucosidic linkages (BARKER, BOURNE and STACEY, 1953).

1.3. OUTLINE OF THE INVESTIGATIONS

When *Arthrobacter*, a bacterium of the *Corynebacteriaceae* family, was cultivated in a carbohydrate-containing medium, deficient in nitrogen, cells were obtained with a high carbohydrate content, the latter in some cases amounting to more than 70 per cent of the dry weight (MULDER et al., 1962). This carbohydrate was found to consist mainly of polysaccharides built up from glucose.

In the first instance the circumstances were studied which influence the formation of these polysaccharides in the cell. Furthermore, a study was made of the functions of these accumulated products in the cell. Attention was particularly paid to the functioning of these polysaccharides as substrates in endogenous respiration and as carbon sources in the synthesis of proteins and consequently in cell growth and cell multiplication.

From a number of *Arthrobacter* strains these polysaccharides have been isolated in pure form, and structural determinations carried out.

In a series of enzymic investigations a study was made of the metabolism of the intracellular polysaccharide of *Arthrobacter*. The structural properties of this *Arthrobacter* polysaccharide were related to the enzyme systems involved in synthesis and breakdown of this carbohydrate.

2. EXPERIMENTAL MATERIAL AND CHEMICAL METHODS

2.1. *ARTHROBACTER* STRAINS

The *Arthrobacter* strains used in this investigation were the numbers 1, 4, 8, 11, 29, 41, 159, 166 of the culture collection of the Laboratory of Microbiology at Wageningen (MULDER and ANTHEUNISSE, 1963). These strains have been isolated from the soil of the laboratory garden. All the strains showed a rod-like appearance in young cultures, but after one or more days the rod form gradually transformed into the coccoid stage. In general the cells were not motile, although strain 1 showed motility under certain conditions.

All the strains grew readily in an inorganic salts medium with $(NH_4)_2SO_4$ as the sole nitrogen compound and glucose as the carbon source. They were colourless, but showed a white glistening appearance when cultivated on a slant of yeast extract – glucose – agar.

According to the classification of BERGEY's Manual (1957) these strains belong to *A. globiformis* when hydrolysing starch and to *A. simplex* when not doing so. Starch hydrolysis was studied by growing the bacteria on starch plates (soluble starch, 1%; glucose, 0.1%; yeast extract, 0.1%) and by flooding the plates after three days with Lugol's iodine solution. Strains 1, 4, 11, 29, and 159 were unable to hydrolyse starch and thus belong to *A. simplex*; strains 8, 41, and 166 gave a positive reaction and thus belong to *A. globiformis*.

Because the non-appearance of the blue colour of the iodine-starch complex is not an unequivocal proof that starch hydrolysis had taken place, (cf. section 7.4.1.) this characteristic was studied once more by inoculating the strains into a liquid medium of the following composition: glucose 0.5%; soluble starch, 0.5%; $(NH_4)_2SO_4$, 0.3%; K_2HPO_4, 0.1%; $MgCl_2$, 0.02%; $CaCO_3$, 0.25%. The disappearance of the glucose and the soluble starch was measured quantitatively. Strain 1 consumed only glucose, but not soluble starch. The cell yield obtained amounted to 2.5 mg of dry weight per ml of culture solution.

Strain 41 (*A. globiformis*), when cultivated in the above-mentioned medium, utilized both glucose and soluble starch. Glucose had disappeared after 20 hours' incubation on a mechanical shaker at 30°; only after this period was soluble starch rapidly consumed. In this case the cell yield was doubled, amounting to 5.2 mg of dry weight per ml of culture solution. Consequently, this strain can use soluble starch as a carbon source.

2.2. SUBSTRATES, ENZYMES, COENZYMES AND REAGENTS

Substrates: soluble starch (Brocades); amylopectin, amylose-free (Calbiochem); amylose (Calbiochem); glycogen (NBC); D(+)glycogen, puriss. (Fluka); uridine-5'-diphosphate glucose, UDPG (Calbiochem); D-glucose-1-phosphate (NBC); D-glucose-6-phospate (Sigma); phosphoenolpyruvic acid (Calbiochem).
Enzymes: uridine diphosphate glucose dehydrogenase (Sigma); pyruvate

kinase (muscle, Calbiochem); α-amylase from *Bacillus subtilis* (Sigma); β-amylase from barley, purum (Fluka); glucose-6-phosphate dehydrogenase, cryst. (Sigma); phosphoglucomutase from rabbit muscle, cryst. (Sigma); hexokinase from yeast, cryst. (Sigma).

Coenzymes: ATP, adenosine-5′-triphosphate (Sigma); UDP, uridine-5′-diphosphate (Calbiochem); NAD, nicotinamide adenine dinucleotide (Gist en Spiritus); NADP, nicotinamide adenine dinucleotide phosphate, cryst. (Sigma).

Reagents: sodium(meta)periodate, puriss. p.a. (Fluka); anthrone, p.a. (Merck); dimethylsulphate, puriss. p.a. (Fluka).

Abbreviations used: Calbiochem: California Corporation for Biochemical Research, Los Angeles; Fluka: Fluka A.G. Buchs S.G. Switzerland; Sigma: Sigma Chemical Company, St.Louis, Missouri, USA; Brocades: Brocades-Stheeman en Pharmacia, Amsterdam; Gist en Spiritus: Ned. Gist- en Spiritusfabrieken, Delft; Merck: Merck A.G., Darmstadt, Germany; NBC: Nutritional Biochemicals Corporation, Cleveland, Ohio, USA.

When an enzyme is mentioned in this investigation for the first time, its systematic name and its code number are given, following the rules of the Commission on Enzymes of the International Union of Biochemistry (cf. the Report of the Commission on Enzymes, 1961). Thereafter the trivial name is used.

2.3. Determination of reducing sugars

Reducing sugars are determined by reacting with alkaline copper reagent. The complex-bound cupric ions are then reduced to insoluble Cu_2O. After that the remaining cupric ions are titrated iodometrically (method of Luff-Schoorl) or the precipitated Cu_2O is determined with the reagent of Nelson, giving the intense blue colour of molybdenum blue (method of Somogyi-Nelson).

2.3.1. *Method of Luff-Schoorl* (Schoorl, 1929).

Reagent of Luff: Copper sulphate ($CuSO_4 \cdot 5H_2O$; 25 g), citric acid ($C_6H_8O_7 \cdot H_2O$; 50 g) and cryst. sodium carbonate ($Na_2CO_3 \cdot 10H_2O$; 388 g) are dissolved in water and made up with water to 1 l. The quantity of cupric ions in this reagent is determined by mixing 25 ml Luff solution with 3 g KI in a 300 ml Erlenmeyer flask, adding 25 ml sulphuric acid (25%) and titrating with 0.1 N sodium thiosulphate, using starch as the indicator.

The sugar determination is carried out as follows: 25 ml of Luff solution is pipetted into an Erlenmeyer flask of 300 ml capacity. The sugar sample (10–50 mg) is added and the mixture is made up to 50 ml. This solution is rapidly heated to the boiling-point and then heated for 10 min under reflux. After cooling, the remaining cupric ions are determined iodometrically and subtracted from the blank. The amount of sugar can be calculated, using the table given by Schoorl.

2.3.2. *Method of Somogyi-Nelson* (Somogyi, 1952).

Reagent of Somogyi: Copper sulphate (4 g), anhydric sodium carbonate (24 g), sodium bicarbonate (16 g), Rochelle salt (12 g) and anhydric sodium

sulphate (180 g) are dissolved in water and the solution made up to 1 l.
Reagent of Nelson: Ammonium molybdate (25 g) is dissolved in 450 ml of water; to this solution is added 21 ml of concentrated sulphuric acid and then a solution of 3 g sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) dissolved in 25 ml of water. Before use this reagent must be incubated for 24–48 h at 37°.
Procedure: 2 ml of copper reagent and 2 ml of sugar solution are mixed in a tube. Samples contain 10–100 μg of sugar. The tubes are heated for 15 min in a boiling-water bath. After cooling, 2 ml of Nelson reagent is added. The absorbancy of the blue colour is measured in a Beckman DU spectrophotometer at 520 mμ against a blank. A standard solution is made, which contains 100 μg of sugar per ml.

2.4. Determination of the total carbohydrate content of the bacterial cell

The determination of the total carbohydrate content of the cell proceeds in two stages: the cells are first hydrolysed with sulphuric acid, after which the liberated reducing sugars are determined. In some cases both steps are carried out in one procedure (anthrone method; phenol-sulphuric acid method).

2.4.1. *Anthrone method* (Trevelyan and Harrison, 1952)

The bacteria are collected by centrifugation and washed once with distilled water. They are resuspended in water and the suspension diluted so that the total carbohydrate content corresponds to an equivalent of 10–100 μg glucose per ml.

The reagent is prepared as follows. To 40 ml of water is added 100 ml of concentrated sulphuric acid. Anthrone (200 mg, Merck) is dissolved in 100 ml of the dilute acid. This reagent can be stored for about two weeks at –15°.
Procedure: 5 ml of reagent is pipetted into tubes and 1 ml of sample, containing 10–100 μg glucose or an equivalent amount of carbohydrate is added and the mixture shaken. After closing the tubes, they are heated for exactly 10 min in a boiling-water bath. Then they are rapidly cooled with tap water. The absorbancy (620 mμ) of the green colour is measured in a 1 cm cuvette of the Beckman spectrophotometer against a blank. A linear relationship exists between the absorbancy of the solution and the glucose concentration. The standard solution contains 100 μg of glucose per tube.

2.4.2. *Phenol-sulphuric acid method* (Dubois et al., 1951).

Reagents: sulphuric acid, p.a., 96%; phenol, 80% (w/v).
Procedure: 2 ml of sugar solution, containing 10–70 μg of sugar is pipetted into tubes and 0.1 ml of 80% phenol added. From a fast delivery pipette 5 ml of concentrated sulphuric acid is added in the course of about 5 sec. After standing for 30 min at room temperature the red colour obtained can be measured at 490 mμ against a blank.

2.4.3. *Method of* DAGLEY *and* DAWES (1949)

A washed bacterial suspension (5 ml, containing 10–50 mg of carbohydrate) is placed in an ampoule and 0.3 ml of concentrated sulphuric acid added (final concentration 2 N H_2SO_4). The opening of the ampoule is sealed and the contents heated in a boiling-water bath for about 10 h. After the hydrolysis, the tubes are cooled, opened, and the solution diluted with water to 25 ml and neutralized with sodium bicarbonate. Reducing sugars are determined according to the method of Luff-Schoorl. The results are expressed as mg of $C_6H_{10}O_5$, the carbohydrate contents as per cent by weight of $C_6H_{10}O_5$ calculated on the dry weight of the cells.

The results obtained by using different methods showed a good agreement. The anthrone and the phenol-sulphuric acid methods have the advantage of being quick procedures, while less than 1 mg of sample is required for the analysis. The latter method has the advantage, that the hydrolysate can be used for a chromatographic determination of the sugars present.

2.5. DETERMINATION OF ALKALI-STABLE POLYSACCHARIDES OF BACTERIA BY THE PFLÜGER METHOD (HASSID AND ABRAHAM, 1957).

The determination of the alkali-stable polysaccharides, which include cell glycogens and cell wall polysaccharides, is carried out as follows. The cells are hydrolysed with a concentrated potassium hydroxide solution, so that the cells completely go into solution, but no hydrolysis of the alkali-stable polysaccharides occurs. The latter are precipitated from the solution by adding ethanol and determined quantitatively (anthrone method).

Bacteria are removed from 10 ml of culture solution by centrifugation and washed once with distilled water. Then 1 ml of 30% KOH is added to the centrifugated sample in the centrifuge tube. The contents of the tube are heated for 1 h at 100° in a water bath until a homogeneous solution is obtained. After cooling, 2 ml of ethanol is added, the mixture shaken and the precipitated polysaccharide collected by centrifugation. The precipitated product is dissolved in 10 ml of water. In this solution the polysaccharide content is determined by the anthrone method.

2.6. PROTEIN DETERMINATION ACCORDING TO LOWREY (1951)

The protein content in bacterial extracts was determined according to Lowrey with the reagent of Folin-Ciocalteu and crystalline serum albumine as the standard. The determination was carried out according to the modification of DEMOSS and BARD (1957).

3. FACTORS INFLUENCING THE FORMATION OF POLYSACCHARIDES BY *ARTHROBACTER*

3.1. TOTAL CARBOHYDRATE CONTENT OF DIFFERENT *ARTHROBACTER* STRAINS AND SOME STRAINS OF BACTERIAL SPECIES OF OTHER GENERA

Bacteria were cultivated in 100 ml medium of the following composition: glucose 1%; yeast extract 0.4%, dissolved in tap water. Cells from two days old agar slants (yeast extract – glucose – agar) were used for inoculation. The experiments were carried out in 300 ml Erlenmeyer flasks, which were incubated on a rotary shaker at 28° for 2 days. Afterwards determinations were carried out of the dry weight and the total carbohydrate content of the cells. The bacteria were harvested by centrifugation, washed once with distilled water and subsequently resuspended in 10 ml of water.

From the results obtained (Table 1) it will be seen, that *Arthrobacter* cells, when cultivated in a medium with a relatively high glucose/yeast extract ratio, possess a high carbohydrate content. Amounts between 50 and 70% of carbohydrate in the dry weight were found in contrast to much lower values in bacteria of different genera.

TABLE 1. Carbohydrate content of a number of *Arthrobacter* strains.

Strain	Dry weight of cells (mg per ml of culture)	Total carbohydrate of cells	
		mg $C_6H_{10}O_5$ per ml	% of dry weight
1	2.8	1.9	68
4	3.4	2.0	59
8	1.5	1.0	67
11	2.7	1.3	50
29	2.4	1.4	60
41	2.0	1.3	65
159	2.6	1.0	38
166	2.5	1.3	54
E.coli	1.1	0.23	21
Bac. cereus	0.3	0.10	33

Upon hydrolysis of these *Arthrobacter* cells with sulphuric acid and chromatographical analysis of the hydrolysate, the major component was found to be glucose; in addition small amounts of other sugars were present.

3.2. CARBOHYDRATE CONTENT OF *ARTHROBACTER*, STRAIN 1, WHEN CULTIVATED WITH DIFFERENT CARBON SOURCES

The carbohydrate content of *Arthrobacter*, strain 1, was estimated after the cultivation of the organism in media supplied with different carbon sources. The composition of the medium was as follows: K_2HPO_4, 4.4; KH_2PO_4, 1.9; $(NH_4)_2SO_4$, 3; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.04 g per l. Trace elements:

$FeCl_3 \cdot 6H_2O$, 2.5; H_3BO_3, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; $CoCl_2 \cdot 6H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.01; $MnCl_2$, 1; $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg per l. The carbon compounds were supplied in amounts of 1%. Cultivation took place at 30° in Erlenmeyer flasks of 300 ml capacity, containing 100 ml of nutrient solution, which were aerated on a rotary shaker for 48 h.

From the data of table 2 it will be seen, that the hexoses and the disaccharides (maltose, lactose and sucrose) supported good growth while the carbohydrate content of the cells was high. The dry weight of the cells amounted to 3–4 mg per ml of culture with the carbohydrate content varying between 40 and 60 per cent. Sorbose was the only hexose not being utilized. The same was true of a number of pentoses; on the other hand, the methyl-pentose rhamnose supported a very good growth. Media in which one of the acids of the tricarboxylic acid cycle was the sole carbon source gave a fair growth, but the polysaccharide content of the cells was considerably lower.

TABLE 2. Effect of the carbon source of the nutrient medium on the carbohydrate content of *Arthrobacter*, strain 1.

Carbon source	Dry weight of cells (mg per ml culture)	Total cell carbohydrates (calculated as $C_6H_{10}O_5$)		Final pH of culture solution
		mg/ml	% of dry weight	
Glucose	3.10	1.29	42	5.6
Mannose	4.40	2.38	54	5.7
Galactose	4.20	1.68	40	5.5
Fructose	2.68	1.01	38	5.2
L-Sorbose	0.0	0.0	–	–
D-Xylose	0.0	0.0	–	–
L-Arabinose	p[1]	–	–	–
L-Rhamnose	4.10	1.67	41	5.3
D-Mannitol	2.30	0.73	32	5.1
D-Sorbitol	0.44	0.10	23	6.6
Galacturonate	0.0	0.0	–	–
Lactose	4.25	2.60	61	5.2
Maltose	3.16	1.67	53	5.4
Sucrose	2.90	1.53	53	3.8
Fumarate	1.05	0.30	29	9.1
Pyruvate	0.60	0.12	20	8.0
Citrate	0.37	0.12	32	8.9
Succinate	2.08	0.43	21	9.0
Malate	1.82	0.37	20	8.6
Acetate	p[1]	–	–	–
Asparagine[2]	1.66	0.46	28	8.9
Aspartate[2]	1.64	0.45	27	9.2
Glutamate[2]	2.38	0.38	16	8.9

[1] p: Very poor growth; [2] No $(NH_4)_2SO_4$ added.

When amino acids served as the sole source of both carbon and nitrogen, relatively good growth of the organism was obtained, but the carbohydrate contents of the cells were low.

In order to investigate if in *Arthrobacter* cells supplied with different carbon compounds, the polysaccharides formed had the same composition, bacteria grown on glucose, mannose, galactose, fructose, rhamnose and mannitol were collected by centrifugation, washed once with distilled water and hydrolysed with 2 N H_2SO_4. The hydrolysates were deionized and subjected to chromatography using n-butanol – acetic acid – water (4:1:5; v/v, upper layer) as a solvent. In all cases the general picture of the chromatogram was the same. The main component was a spot, having a R_F-value of 0.16, which was identified as glucose. In addition a number of faint spots were present, two of which were identified with certainty as rhamnose and mannose.

3.3. Carbohydrate content of *Arthrobacter*, strain 1 at different stages of growth

Arthrobacter, strain 1, was cultivated in a medium, containing 0.4% yeast extract and 1% glucose in tap water. Cells used for inoculation were precultivated in 100 ml of the same medium in an Erlenmeyer flask of 300 ml capacity. After 24 h incubation at 30° the contents of one flask were added to 1 l of this medium in a 5 l Erlenmeyer flask. This flask was aerated on a mechanical shaker at 30°. Samples were withdrawn at different time intervals. In these samples the dry weight of the cells, their carbohydrate content and the glucose concentration of the medium were determined (Table 3; Figure 1).

Table 3. Effect of incubation time of *Arthrobacter*, strain 1, on yield and carbohydrate content of the cells.

Time (h)	Dry weight of cells	Cell carbohydrates		Glucose in medium
	(mg per ml culture)	mg/ml	% of dry weight	mg per ml
0				9.3
4	0.57	0.2	35	9.0
8	0.91	0.3	33	8.5
13	1.52	0.6	40	7.1
24	2.5	1.2	48	4.0
32	3.6	2.3	64	1.5
47	4.3	2.5	62	0.0
73	3.6	2.0	55	0.0
120	3.4	1.8	53	0.0[1]

[1]pH at the end of the experiment was 6.2.

Growth proceeded roughly linearly with time as did the decrease of the glucose concentration in the medium. This apparently depended on sub-optimal aeration of the culture medium, as may be concluded from the following considerations. The oxygen uptake capacity of the culture medium at a given shaking intensity is constant. When the oxygen consumption by the bacteria becomes equal to this oxygen uptake capacity, then the growth rate also becomes constant. In a Kluyver flask, where air is bubbled through the medium, growth

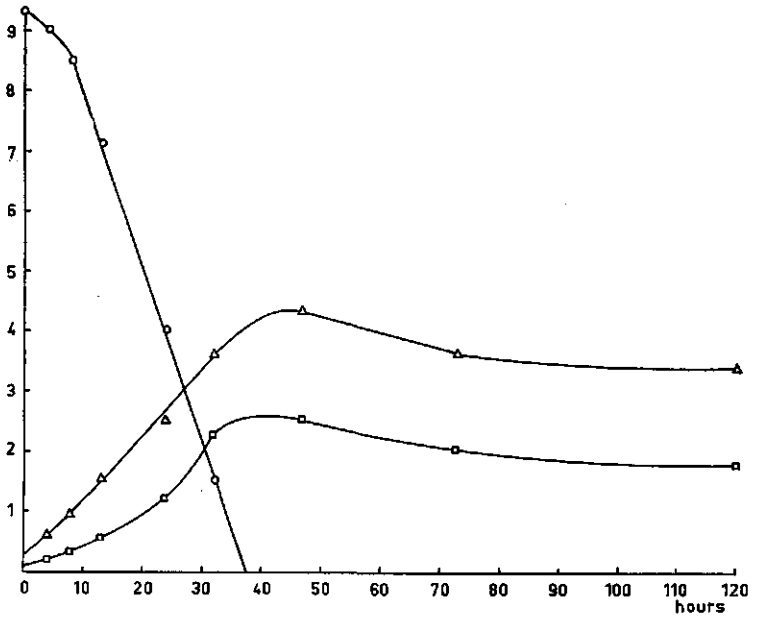


FIGURE 1. Effect of time of incubation of *Arthrobacter*, strain 1, on yield and carbohydrate content of the cells (mg/ml of culture).
△, dry weight of cells; □, amount of cell carbohydrate; ○, glucose in the medium.

is not limited by the entry of oxygen and as a result of this it proceeded exponentially (cf. section 3.4.).

During the first 12 h of the growing period the carbohydrate content approximated 30–35%, but at the end of the growth phase it had almost doubled (Table 3). This sharp rise of the carbohydrate content was due to exhaustion of nitrogen whereas glucose was still present in the nutrient solution in relatively large amounts. Synthesis of protein came to a standstil and the glucose taken up by the bacteria was converted to a large extent into polysaccharides which accumulated within the cells (cf. the increase of the dry weight and that of carbohydrate content of the cells at 24 and 32 h). At about 47 h the glucose had disappeared from the medium and maximal cell yield was obtained. Upon continuation of the aeration, a slight decrease in the dry weight of the cells and their carbohydrate content took place from 64 to 53 per cent within approximately 3 days. This decrease of dry weight was due to the utilization of polysaccharides in the endogenous respiration of the cells (cf. section 4.2.).

3.4. EFFECT OF PH OF THE NUTRIENT MEDIUM ON THE CARBOHYDRATE CONTENT OF *ARTHROBACTER*

In the experiments pertaining to the effect of pH, *Arthrobacter*, strain 1, was cultivated in the basal medium (for composition see section 3.2.), supplied

with 1% glucose. The cells were cultivated in a Kluyver flask at 28°. Samples were withdrawn from the culture solution at different periods of time. The following estimations were carried out in these samples: a. dry weight, b. total carbohydrate, c. polysaccharides of the cells, d. glucose concentration and e. pH of the culture medium.

Table 4 and Fig. 2 give the result of an experiment in which the pH of the culture solution was buffered inadequately. In the course of the first 12 h the pH of the medium dropped to 4.5. Hereafter there was no further increase of acidity. At the end of this period the glucose had not yet disappeared from the nutrient solution, but growth and glucose consumption had slowed down. The polysaccharide content of the cells increased, particularly from the 6th to the 12th hour when the pH dropped from 6.3 to 4.5. Hereafter the dry weight of the cells increased only slowly while the carbohydrate content remained constant. After 50 h there was a sudden drop of these values, owing to the disintegration of the cells by autolysis. This was verified by microscopical examination of the culture and by chemical analysis of the medium.

TABLE 4. Effect of time of incubation on yield and carbohydrate content of *Arthrobacter*, strain 1, grown in an inadequately buffered culture solution.

Time (h)	Dry weight of cells	Cell carbohydrates (anthrone method)		Cell poly-saccharides (Pflüger method)	Glucose in medium	pH
	mg/ml	mg/ml	% of dry weight	mg $C_6H_{10}O_5$/ml	mg/ml	
0						7.4
3	0.65	0.14	22	0.10	10.0	6.6
6	1.24	0.23	19	0.18	7.9	6.3
8.5	2.70	0.76	28	0.60	4.8	5.2
11.5	4.04	1.9	47	1.9	2.5	4.5
15	4.07	2.0	49	2.1	2.0	4.6
25	4.28	2.2	51	2.2	1.1	4.4
50	4.89	2.5	51	2.5	0.5	4.5
72	3.39	1.3	38	1.3	0.3	4.6
96	2.80	0.9	32	0.7	0.2	4.7

In this experiment it was furthermore found that the amount of cell polysaccharides, determined according to the Pflüger method, corresponded to the total carbohydrate content of the cell as determined by the anthrone method (Table 4).

The drop in pH of the nutrient solution was caused in the first place by withdrawal of NH_4 ions from the medium, resulting in free sulphuric acid; furthermore by excretion of organic acids into the medium. The latter were isolated by ether extraction of the medium after acidifying with sulphuric acid. The acids were separated by chromatography using n-butanol – formic acid – water as a solvent. Glucose-aniline, dissolved in ethanol – butanol – water mixture, was applied as a spray. In this way several spots of non-volatile organic acids were obtained, α-ketoglutaric acid being the most prominent.

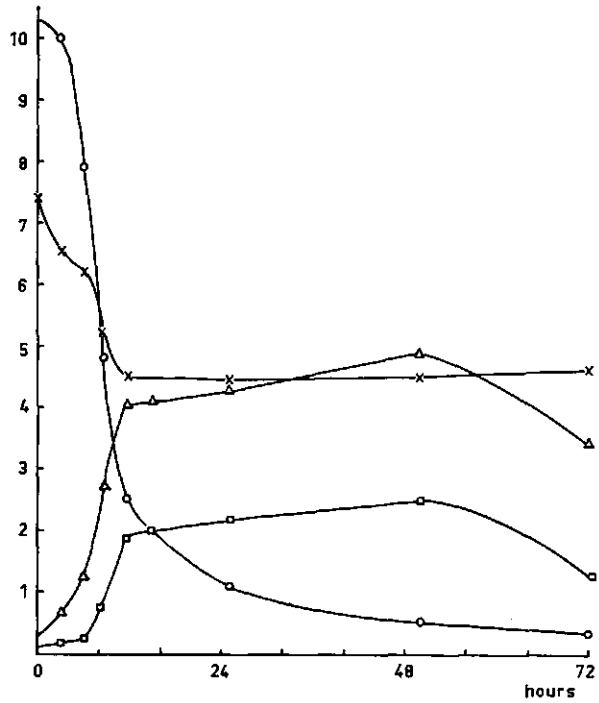


FIGURE 2. Effect of time of incubation on yield and carbohydrate content (mg/ml of culture) of *Arthrobacter*, strain 1, grown in an inadequately buffered culture solution.
△, dry weight of cells; □, amount of cell carbohydrate; ○, glucose in the culture medium (mg/ml); ×, pH of the medium.

In a subsequent experiment the pH of the medium was kept constant by titrating the contents of the Kluyver flask with sterile NaOH from a dropping funnel. Bromothymol blue was used as an indicator (pH-range 6.0–7.6). In this way the pH of the culture solution was kept between 6 and 7 (cf. Table 5 and Figure 3). This procedure required a continuous visual control of the contents of the flask, while another disadvantage was, that bromothymol blue was slowly broken down by the bacteria.

The composition of the medium was the same as that employed in the preceding experiment; the temperature was 28°. After 14 h the glucose had disappeared from the medium. The cells then arrived in the stationary phase,

TABLE 5. Yield and carbohydrate content of *Arthrobacter*, strain 1, when grown in a culture solution kept at constant pH by titrating with NaOH.

Time (h)	Dry weight of cells mg/ml of culture	Carbohydrate content of cells		Glucose in medium mg/ml	pH of medium
		mg/ml of culture	% of dry cell weight		
0	0.48	0.11	23	11.1	6.6
5	1.12	0.29	26	10.6	6.6
8.5	3.14	0.81	26	5.88	6.6
11	5.09	1.51	30	2.12	6.2
14.5	5.81	1.64	28	0.0	6.6
25.5	5.62	1.37	24	0.0	6.8
50	5.92	1.30	22	0.0	7.0

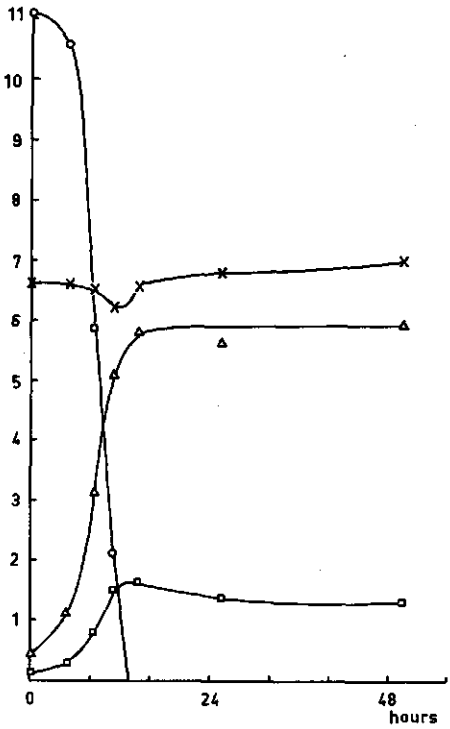


FIGURE 3. Yield and carbohydrate content (mg/ml of culture) of *Arthrobacter*, strain 1, grown in a culture solution kept at constant pH by titrating with NaOH.
△, dry weight of cells; □, cell carbohydrates; ○, glucose in the culture medium (mg/ml); ×, pH of the medium.

showing the coccoid form. From the data of table 5 it will be seen that the carbohydrate content of the cells was much lower than in the preceding experiment; the maximum value was 30 per cent, which gradually decreased to 22 per cent after 50 h.

In a corresponding experiment the pH was kept constant by adding $CaCO_3$ to the medium. The composition of the culture solution was as follows: glucose, 10; $(NH_4)_2SO_4$, 3; K_2HPO_4, 1; $MgCl_2$, 0.2; $CaCO_3$, 2.5 g; trace elements as usual; distilled water 1000 ml.

TABLE 6. Yield and carbohydrate content of *Arthrobacter*, strain 1, when grown in a culture solution kept at constant pH by added $CaCO_3$.

Time (h)	Dry weight of cells mg/ml of culture	Carbohydrate content of cells		Glucose in medium mg/ml	pH
		mg/ml culture	% of dry weight		
0	0.06	–	–	9.48	6.9
5	0.34	–	–	9.00	6.7
8.25	1.07	0.22	21	7.08	6.6
12	2.20	0.34	16	3.74	6.4
14.5	3.55	0.68	19	0.48	6.2
24	4.22	1.09	26	0.0	6.3
49	4.20	1.03	25	0.0	6.5
77	4.34	0.99	23	0.0	6.6

The pH of the culture medium remained between 6.2 and 6.9 (Table 6; Figure 4). The carbohydrate content of the cells remained low (below 26%) and this decreased slowly after glucose had disappeared from the medium.

The results of the foregoing experiments show, that the pH of the nutrient medium had a pronounced effect on the formation of intracellular polysaccharides. When *Arthrobacter*, strain 1, was growing in an inorganic salts medium, buffered with 0.7 per cent phosphate buffer and with glucose as the carbon source, the pH of the medium decreased rapidly in spite of the buffer capacity of the medium. Cells were obtained with a high carbohydrate content. By growing the cells in the same medium, the pH of which was maintained at a neutral value by titration with NaOH, the carbohydrate content of the cells remained low. The same effect was obtained by adding $CaCO_3$ to the medium.

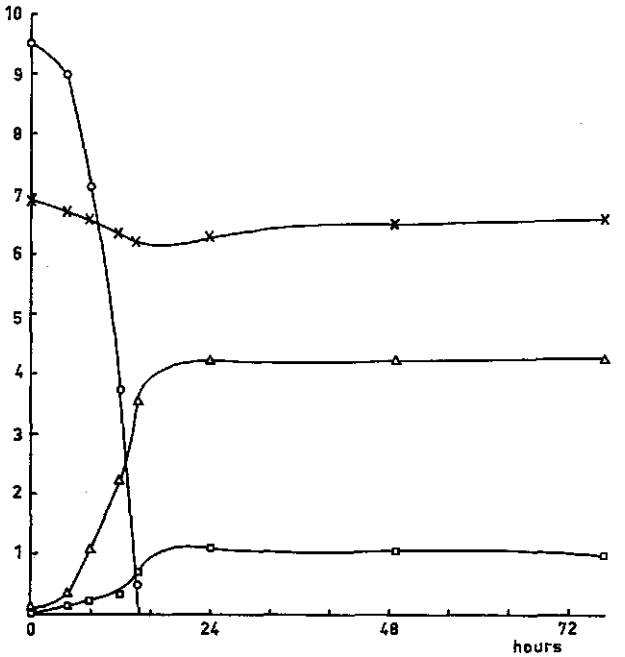


FIGURE 4. Yield and carbohydrate content (mg/ml of culture) of *Arthrobacter*, strain 1, grown in a culture medium kept at constant pH by added $CaCO_3$.
△, dry weight of cells; □, cell carbohydrates; ○, glucose in the culture medium (mg/ml); ×, pH of the medium.

3.5. INFLUENCE OF NITROGEN DEFICIENCY ON THE CARBOHYDRATE CONTENT OF *ARTHROBACTER*, STRAIN 1

Arthrobacter, strain 1, was cultivated in a medium of the following composition: glucose, 10; K_2HPO_4, 1; $MgCl_2$, 0.2; $CaCO_3$, 2.5 g; trace elements as usual; distilled water 1000 ml; $(NH_4)_2SO_4$ was supplied in increasing concentrations, viz. 0.05%, 0.1%, 0.2% and 0.3%.

Cells from two days old agar slants (yeast extract – glucose – agar) were suspended in sterile water and 1 ml of this suspension transferred into 100 ml of the above medium contained in 300 ml Erlenmeyer flasks. The cells were cultivated for 2 days at 30° on a mechanical shaker.

The amount of cell proteins formed, depended on the quantity of $(NH_4)_2SO_4$ in the medium. When all the nitrogen was used up and glucose was still present, the cells converted part of the excess of glucose into intracellular polysaccharide (up to 70% of the dry weight; table 7).

TABLE 7. Effect of increasing amounts of $(NH_4)_2SO_4$ at a constant glucose level on dry weight and carbohydrate content of *Arthrobacter*, strain 1.

$(NH_4)_2SO_4$ in medium (%)	Dry weight of cells mg/ml of culture	Cell carbohydrates		Carbohydrate-free cell material mg/ml	Glucose[1] in medium
		mg/ml of culture	% of dry weight		
0.05	1.90	1.24	65	0.66	++
	1.84	1.18	64	0.66	++
	1.95	1.38	71	0.57	++
	2.02	1.47	73	0.55	++
0.10	2.54	1.32	52	1.22	+
	3.66	2.43	66.5	1.23	±
0.20	3.86	1.45	37.5	2.41	—
	3.90	1.48	38	2.42	—
0.30	4.89	1.44	29	3.45	—
	4.20	1.03	25	3.17	—

[1]Glucose in the medium at the end of the experiment; ++, strongly, and +, slightly positive; —, no reaction.

The amounts of carbohydrate-free cell material were derived by subtracting total carbohydrate from the dry weight of the cells (Table 7). This material was assumed to consist principally of proteins. The quantity of protein calculated in this way was equal to the theoretical amount based on the amount of nitrogen available (assuming the nitrogen content of the bacterial protein to be 16 per cent).

The minimum amount of carbohydrate found in the cells amounted to about 25–30% of the dry weight. This value was obtained with 0.3% $(NH_4)_2SO_4$. Under these conditions values of 3.0–3.5 mg of carbohydrate-free cell material and 4–5 mg dry cell material were obtained from 10 mg glucose which may be considered as optimum yields.

3.6. INFLUENCE OF PHOSPHORUS AND SULPHUR DEFICIENCIES ON THE CARBOHYDRATE CONTENT OF *ARTHROBACTER*, STRAIN 1

3.6.1. *Effect of increasing phosphate concentrations*

The composition of the nutrient medium was as follows: glucose, 10; $(NH_4)_2SO_4$, 3; K_2SO_4, 1; $MgCl_2$, 0.2; $CaCO_3$, 2.5 g/l; trace elements as usual; K_2HPO_4 in increasing amounts of 50, 100, 200, 300 and 500 mg per l. The

flasks were inoculated with 1 ml of a suspension of *Arthrobacter*, strain 1, grown for 2 days on a yeast extract – glucose – agar, and cultivated for 2 days on a rotary shaker.

From the results of this experiment (Table 8) it will be seen that phosphorus deficiency effected carbohydrate content similarly to nitrogen deficiency. At low phosphate concentrations growth was restricted whereas the carbohydrate content of the cells was high; at the end of the experiment glucose was not used up. At higher phosphate concentrations, the yield of cells (dry weight) was higher and glucose was used up completely; 0.05% K_2HPO_4 was sufficient for optimal growth. At this concentration the carbohydrate content of the cells was much lower.

TABLE 8. Effect of increasing amounts of K_2HPO_4 at a constant glucose level on dry weight and carbohydrate content of *Arthrobacter*, strain 1.

K_2HPO_4 in medium (%)	Dry weight of cells mg/ml of culture	Cell carbohydrates		Glucose in medium[1]
		mg/ml of culture	% of dry weight	
0.005	2.00	1.25	62.5	++
	1.96	1.23	63	++
0.01	3.30	2.02	61	+
	3.26	1.97	60.5	+
0.02	4.04	2.14	53	+
	3.91	2.06	53	—
0.03	3.89	1.48	38	—
0.05	3.81	1.27	33	—

[1]Glucose in the medium at the end of the experiment; ++, strongly, and +, slightly positive; —, no reaction.

3.6.2. *Effect of increasing sulphate concentrations*

The composition of the medium used in this experiment was as follows: glucose 10; NH_4Cl, 3; K_2HPO_4, 1; $MgCl_2$, 0.2; $CaCO_3$, 2.5 g/l; trace elements as usual; K_2SO_4 in increasing concentrations of 10, 20, 40, 50 and 100 mg per l.

TABLE 9. Effect of increasing amounts of K_2SO_4 at a constant glucose level on dry weight and carbohydrate content of *Arthrobacter*, strain 1.

K_2SO_4 in medium (%)	Dry weight of cells mg/ml of culture	Cell carbohydrates		Glucose in medium[1]
		mg/ml of culture	% of dry weight	
0.001	1.32	0.65	49	++
0.002	2.43	1.21	50	++
0.004	3.00	1.22	41	+
0.005	2.70	0.87	32	—
0.010	3.05	0.90	30	—

[1]Glucose in the medium at the end of the experiment; ++, strongly, and +, slightly positive, —, no reaction.

The results obtained with increasing amounts of sulphate resemble those of the experiments with nitrogen and phosphate, although the effect of sulphur deficiency was not as pronounced as in the case of nitrogen or phosphorus deficiency (Table 9).

3.7. Discussion

Intracellular polysaccharide accumulation may take place when growth is inhibited, but glucose utilization and energy production are not disturbed. Growth inhibition may be caused by nutrient depletion of some inorganic nutrient, like nitrogen, resulting in glycogen accumulation.

Holme and Palmstierna (1956) found that during periods of nitrogen limitation resting cells of *Escherichia coli* synthesize and accumulate intracellularly a polyglucose compound resembling glycogen. The same was found in *Aerobacter aerogenes* (Strange et al., 1961) and in *Agrobacterium tumefaciens* (Madsen, 1961). The glycogen-containing cells of *Aerobacter aerogenes* survived extended periods of nutrient limitation longer than cells without glycogen (Strange et al., 1961). Nitrogen-limited stationary cells of *Aerobacter aerogenes* were found to accumulate glycogen in amounts frequently exceeding 20% of the cell dry weight (Segel et al., 1965). The same was true of sulphur-limited cells of *A. aerogenes*. Little or no glycogen accumulation was found in phosphate-limited cells. In addition to nutrient depletion, accumulation may be brought about by growth inhibition caused by other factors. Segel et al. (1965) showed the effect of adding chloramphenicol to log-phase cultures of *A. aerogenes* growing in a complete medium. Immediately upon the addition of the antibiotic exponential growth ceased and glycogen accumulated.

4. FUNCTION OF THE INTRACELLULAR POLYSACCHARIDE OF *ARTHROBACTER*

In the course of the present investigation it was found, that *Arthrobacter* cells are able to survive for a considerable period of time in the absence of an external nutrient supply. This indicates that the cells have at their disposal an intracellular source of reserve material, which provides the energy and carbon substrates necessary for survival. Endogenous metabolism is defined as the sum of the metabolic reactions, taking place in the living cell, when exogenous substrates are absent (DAWES and RIBBONS, 1962a). This endogenous metabolism manifests itself in the endogenous respiration, which serves as the source of energy for the cells to survive (energy of maintenance). Energy is required for osmotic regulation, maintenance of intracellular pH-value, and for motility. In the cell a continuous breakdown of proteins and of nucleic acids occurs; these cell materials have to be resynthesized (turnover of proteins and nucleic acids) and for these processes energy is required. The endogenous substrates also provide carbon substrates for resynthesis of the degraded cellular constituents (LAMANNA and MALETTE, 1959).

Endogenous substrates demonstrated in microorganisms are: carbohydrates (glycogen and other polyglucose compounds), lipids, poly-β-hydroxybutyric acid, peptides, proteins, amino acids, RNA and inorganic polyphosphate (volutin).

In *Saccharomyces cerevisiae* carbohydrates (glycogen and trehalose) serve as endogenous substrates (EATON, 1963).

In *Aerobacter aerogenes* glycogen was recognized as an endogenous reserve (STRANGE, DARK and NESS, 1961) and its presence in the cell favours survival. After glycogen had been utilized, further degradation of proteins occurred and to a lesser extent degradation of RNA commenced, but the residual (structural) carbohydrate was not utilized.

HOLME and PALMSTIERNA (1956) investigated the role of a glycogen-like alkali-stable polysaccharide as a reserve material in *Escherichia coli* B. This glycogen was found to serve as a carbon source for the synthesis of nitrogenous materials (proteins). Furthermore it was found, that the last formed glycogen in the cell was the first to be degraded.

DAWES and RIBBONS (1962) investigated the role of carbon and nitrogen compounds as endogenous substrates in *Escherichia coli*. When washed suspensions of cells were aerated, Q_{O_2} and cellular glycogen decreased to low values during the first few hours. However, when the glycogen had been utilized, release of amino acids and of NH_3 began; during this period the Q_{O_2} remained constant at a low level. They concluded that glycogen served as the primary endogenous substrate and that only when this material had been exhausted, the degradation of other compounds occurred.

MACRAE and WILKINSON (1958) demonstrated the breakdown of poly-β-hydroxybutyric acid in washed suspensions of *Bacillus megaterium* during

aeration. They also reported the degradation of this compound in *Bacillus cereus* under aerobic conditions.

In an experiment with *Sphaerotilus natans*, poly-β-hydroxybutyric acid and polysaccharides were found to occur in large amounts within the cells, particularly in media poor in available nitrogen. When the exogenous substrates had been exhausted and incubation of the cells was continued, a ready respiration of the poly-β-hydroxybutyric acid was observed (MULDER and VAN VEEN, 1963).

The endogenous substrates of *Sarcina lutea* vary with the composition of the medium in which the cells have grown. Peptone-grown cells utilize the amino acid and peptide pools, while carbohydrate and fat content of the cells remained constant. Cells grown in a medium with glucose and peptone also oxidize a hot water soluble polyglucose (DAWES and HOLMS, 1958).

When washed suspensions of *Pseudomonas aeruginosa* were shaken aerobically, NH_3 was released, without loss of viability. The protein, fat, carbohydrate, RNA and DNA contents of the cells did not change, so it was concluded that the free amino acid pool was the source of the endogenous substrates (WARREN, ELLS and CAMPBELL, 1960).

DEINEMA (1961) investigated the importance of both intra- and extracellular lipids of yeast species (*Lipomyces starkeyi* and *Rhodotorula* species) on the longevity of the yeast cells. In the absence of available carbon and nitrogen compounds in the nutrient medium both lipids were used up by the cells. During the period of lipid consumption the number of viable cells did not much vary but after the lipids had disappeared the viability of the cells began to decrease.

In a subsequent experiment DEINEMA (cf. MULDER et al., 1962) added ammonium sulphate to yeast cells containing large amounts of intracellular lipids. A rapid utilization of the reserve material took place resulting in an approximately 10-fold increase of number of cells.

4.1. FORMATION OF INTRACELLULAR POLYSACCHARIDE BY WASHED CELLS OF *ARTHROBACTER*, STRAIN 1

Arthrobacter, strain 1, was precultivated in an inorganic salts medium with glucose as the carbon source. The cells were harvested by centrifugation, washed with distilled water and resuspended in 0.1 % K_2HPO_4. The dry weight of the cells in this suspension amounted to 3.59 mg per ml. Portions of 1 ml were placed in Warburg vessels and oxygen uptake and carbon dioxide production measured with and without 2.0 mg of glucose as the substrate. In addition, a carbohydrate balance of the suspension was made at the beginning and at the end of the experiment.

The results of this investigation show (Figure 5) that at approximately 1 h after the addition of glucose, the respiration rate of the cells supplied with this substrate dropped to a value only slightly higher than that of the endogenous respiration of the control cells. The point of inflexion apparently denotes the exhaustion of the external substrate. A carbohydrate analysis of the cells at the beginning and the end of the experiment is given in table 10.

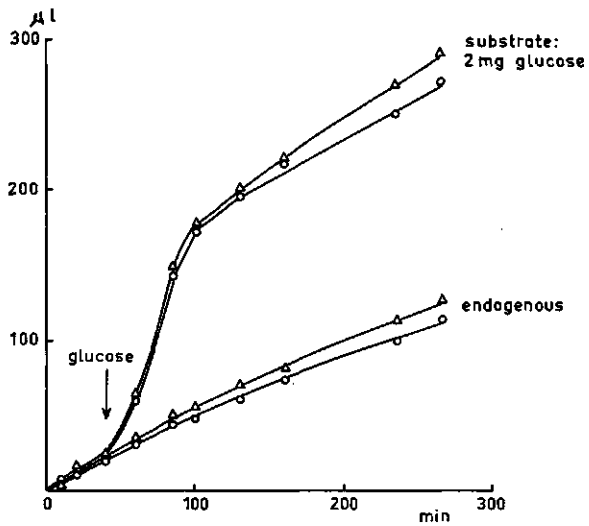


FIGURE 5. Oxygen uptake and carbon dioxide evolution of a washed suspension (1 ml) of *Arthrobacter*, strain 1, after addition of 2 mg glucose.
△, oxygen uptake;
○, carbon dioxide evolution.

Glucose had disappeared from the solution at the end of the incubation period. In the course of the experiment 0.39 mg of glucose (20% of the added compound) had been respired. Simultaneously approx. 1.0 mg (50%) of the added glucose was laid down as glucose polymers. The remaining part (approx. 30%) was not recovered in the carbohydrate balance. This part of the glucose had probably been converted into other polymeric compounds or had been excreted into the medium as organic acids.

TABLE 10. Cell carbohydrate (calculated as glucose) of 1 ml suspension of *Arthrobacter*, strain 1, during the Warburg experiment.

	No glucose added	2 mg glucose added
At zero time	1.52	1.52[1]
After 5 h	1.30	2.57[1]
Respired	0.17	0.39[2]

[1] Determined by the anthrone method
[2] Calculated from the Warburg experiment

4.2 INTRACELLULAR POLYSACCHARIDE OF *ARTHROBACTER*, STRAIN 1, AS SUBSTRATE FOR ENDOGENOUS RESPIRATION

Arthrobacter, strain 1, was cultivated in the basal salts medium with glucose as the sole carbon source (glucose, 1%; $(NH_4)_2SO_4$, 0.1%); subsequently the cells were washed and resuspended in 0.1 M phosphate buffer of pH 7.0. Aliquots of 1 ml of this suspension (dry weight of the cells: 9.44 mg, carbohydrate content: 5.6 mg, calculated as glucose) were pipetted into Warburg vessels and oxygen uptake and carbon dioxide release measured for a period of 70 h at 30°. In additional aliquots of the bacterial suspension, aerated under identical condi-

TABLE 11. Oxygen consumption, carbon dioxide evolution and carbohydrate consumption by a washed suspension of *Arthrobacter*, strain 1.

Time (h)	µl O_2	µl CO_2 (x)	R.Q.	A–x	Carbohydrate of bacterial suspension		
					As mg glucose	As µl CO_2	Decrease (y, as µl CO_2)
0	0	0	–	1800	5.60	4180	0
2	112	114	0.98	1686	5.25	3920	260
4	190	180	0.95	1620	5.15	3840	340
8	344	322	0.94	1478	4.85	3620	560
21	780	760	0.97	1040	4.45	3320	860
52	1440	1340	0.93	460	3.70	2760	1420
70	1620	1500	0.93	300	3.50	2620	1560
124		1800[1]			3.30	2460	1720

[1]A, by extrapolation

tions in a shake flask, carbohydrate determinations were carried out. The results of this experiment are given in table 11 and plotted in figure 6. It will be seen that the amount of polysaccharide disappearing from the cells was equivalent to the amount of carbon dioxide evolved in the Warburg experiment. The R.Q. had a constant value of about 0.95. This demonstrates that carbohydrate is the endogenous substrate. The total quantity of substrate available for endogenous respiration was calculated by extrapolation of the carbon dioxide curve to a time at which the evolution of this gas appeared negligible (Figure 6). This amount approximated to 1800 µl CO_2 (A in table 11). Within 5 days the carbo-

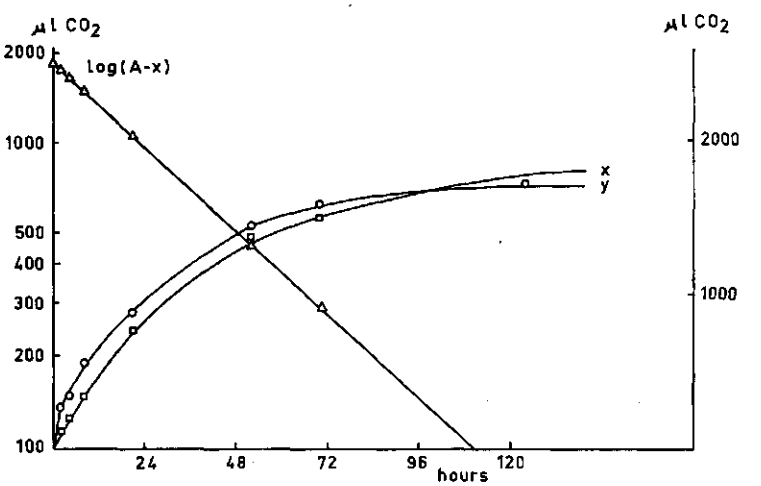


FIGURE 6. Carbon dioxide evolution (x) and carbohydrate consumption (y) as µl CO_2 of a washed suspension (1 ml) of *Arthrobacter*, strain 1.
A is total amount of endogenous substrate, expressed as µl CO_2, found by extrapolation of curve x.

hydrate content of the suspension had decreased from 5.6 to 3.3 mg glucose per ml, corresponding to a quantity of 1720 µl CO_2 (= 2.3 mg glucose). At this time, when 40% of the total carbohydrate had been consumed, the endogenous respiration had arrived at a very low level.

The results of this experiment show that the carbohydrates of the cells tested belonged to two fractions, viz. one (40%), serving as the endogenous substrate and a second (60%), which was not or very slowly broken down.

From a kinetic analysis of the carbon dioxide production it was concluded that only one endogenous substrate was present. If it is assumed, that a total amount of 1800 µl CO_2 can be formed from the endogenous substrate, a plot can be made of the logarithm of the remaining amount A-x against time. A straight line was obtained, indicating that only one endogenous substrate was present disappearing by a first order reaction (EATON, 1959; Figure 6).

The velocity constant of a first order reaction is given by the equation $K = 1/t \ln A/A-x$ so that $\log (A-x) = \log A - K't$, in which A = total endogenous substrate (as CO_2) at zero time, x = carbon dioxide evolved and A–x is the remaining substrate (as CO_2) after time t.

When this kinetic analysis of substrate disappearance was applied to the endogenous respiration of yeast, EATON (1963) found two substrate components disappearing under aerobic conditions, each in accordance with first order reaction kinetics. However, chemical analysis showed that only glycogen-like compounds disappeared from the cell, the amount being equivalent to the amount of carbon dioxide produced. It was concluded, therefore, that there were two glycogen components serving as endogenous substrates. Eaton succeeded in separating these two glycogen components in the ultracentrifuge into a 'light' and a 'heavy' fraction (cf. p. 40).

4.3. INTRACELLULAR POLYSACCHARIDE OF *ARTHROBACTER*, STRAIN 1, FUNCTIONING AS A CARBON SOURCE IN THE SYNTHESIS OF PROTEINS

Arthrobacter, strain 1, was cultivated in 1 l of the basal salts medium deficient in nitrogen (0.1% $(NH_4)_2SO_4$) and supplied with 1% glucose as the carbon source; other constituents as usual. Growth took place in a Kluyver flask at 30°. The cells were harvested at the end of the exponential growth phase and resuspended in 500 ml medium, containing 0.1% K_2HPO_4, 0.02% $MgCl_2$ and trace elements as usual. This suspension was divided into two parts, which were placed in Erlenmeyer flasks of 500 ml capacity. To one of the flasks 1 g $(NH_4)_2SO_4$ was added. The cultures were aerated at 30° on a mechanical shaker. Samples of 10 ml were withdrawn after different periods of time. In these samples the amounts of cell protein and of cell carbohydrate were determined in the following way: cells were collected by centrifugation and resuspended in 5 ml of water. They were then disintegrated for half an hour by ultrasonic vibration. Subsequently the protein content (according to Lowrey) and the carbohydrate content (anthrone method) of the extracts were estimated.

As can be seen from figure 7, the protein content of the cells remained constant

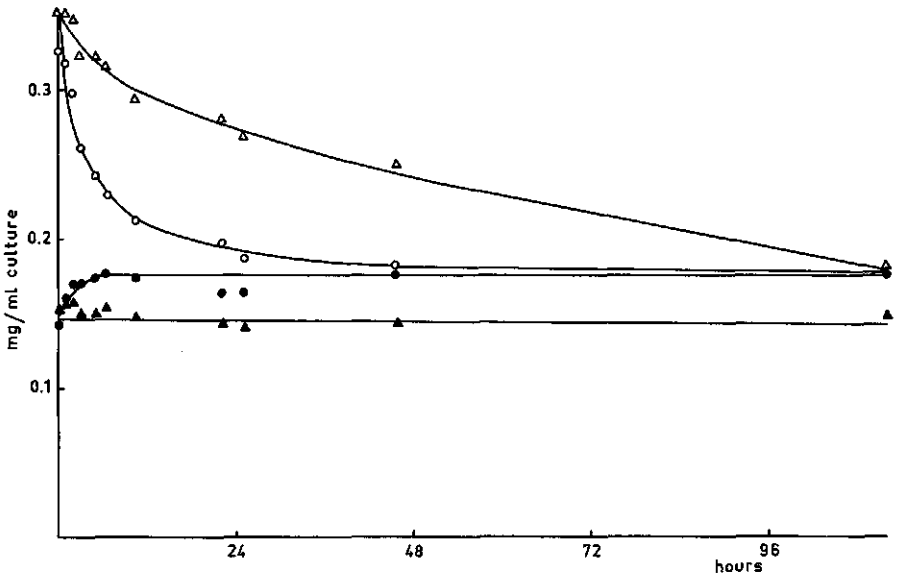


FIGURE 7. Cell carbohydrate and cell protein of a washed suspension of *Arthrobacter*, strain 1, aerated in the presence and in the absence of $(NH_4)_2SO_4$. Open symbols, carbohydrate (△, no; ○, with $(NH_4)_2SO_4$ added). Closed symbols, protein (▲, no; ●, with $(NH_4)_2SO_4$ added).

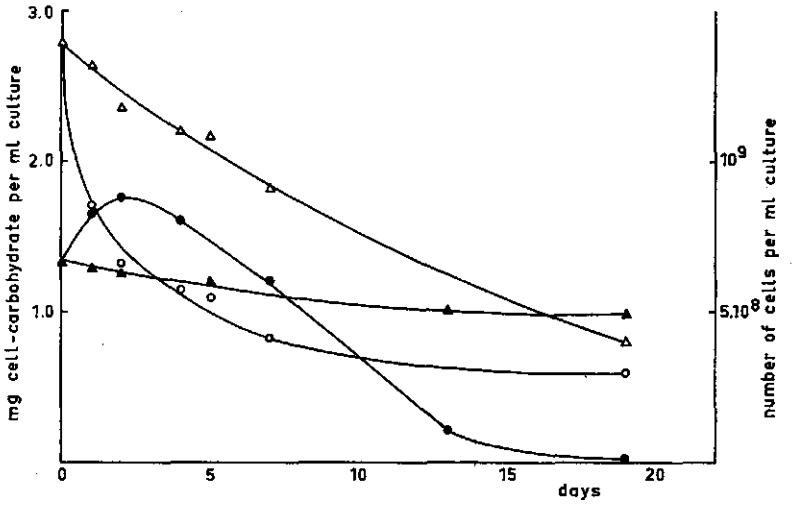


FIGURE 8. Cell carbohydrate and number of viable cells of a washed suspension of *Arthrobacter*, strain 1, aerated in the presence and in the absence of $(NH_4)_2SO_4$. Open symbols, carbohydrate (△, no; ○, with $(NH_4)_2SO_4$ added). Closed symbols, number of viable cells (▲, no; ● with $(NH_4)_2SO_4$ added).

without added $(NH_4)_2SO_4$ and increased with 20% when $(NH_4)_2SO_4$ had been added. In the latter case the carbohydrate content of the cells, which was approx. 70% of the dry weight, fell much more rapidly than in the suspension without added $(NH_4)_2SO_4$. The pH at the end of the experiment was 7.4 without added $(NH_4)_2SO_4$ and 6.7 when $(NH_4)_2SO_4$ had been added.

4.4. Influence of the carbohydrate content of *Arthrobacter*, strain 1, on the longevity of the cells

This experiment was carried out similarly to the preceding experiment, but instead of the protein content the number of viable cells was estimated and related to the carbohydrate content of the cells.

Cell counts were carried out by the plating technique using casein agar (composition: $Ca(H_2PO_4)_2$, 0.025%; $MgSO_4$, 0.025%, $(NH_4)_2SO_4$, 0.025%, K_2HPO_4, 0.1%; glucose, 0.1%; casein, 0.1%; yeast extract, 0.07%; Davis agar, 1%.).

As can be seen from figure 8 the number of viable cells in the flask with added $(NH_4)_2SO_4$ increased during the first two days, but thereafter a pronounced reduction of the number of viable cells occurred. In the absence of added $(NH_4)_2SO_4$ the decrease of the carbohydrate content of the cells was much slower, while the viability of the cells remained constant during the same period of time.

5. ISOLATION AND PURIFICATION OF THE INTRACELLULAR POLYSACCHARIDE OF *ARTHROBACTER*

Several methods of isolating the intracellular polysaccharide of *Arthrobacter* are described in this chapter. For obtaining a high polysaccharide content, the cells were cultivated in a medium with low nitrogen content. The inorganic salts medium had the same composition as usual (cf. section 3.5.) but contained 0.1 % instead of 0.3 % $(NH_4)_2SO_4$. The bacteria were precultivated in 100 ml of this medium in an Erlenmeyer flask of 300 ml capacity by shaking for one day on a mechanical shaker at 28–30°. This culture was then transferred into 1 l of the same medium, contained in a Kluyver flask and cultivated for 24 h, after which the glucose had mostly entirely disappeared. The aeration was stopped, so that the calcium carbonate settled down, the culture decanted, and the cells harvested by centrifugation. Subsequently they were resuspended in about 50 ml of water.

In order to isolate the intracellular polysaccharide it was necessary to disintegrate the cells. This may be done in several ways, viz. a. chemically, by treating the cells with concentrated alkali, and b. mechanically, by exposing them to ultrasonic vibration or by shaking a cell suspension with glass beads.

5.1. Isolation according to the method of Pflüger (Somogyi, 1957)

An equal volume of 20 % KOH is added to the cell suspension, whereupon the mixture is heated on a water bath at 100° for one hour. By this treatment proteins, nucleic acids and fats are hydrolysed. The clear brown solution so obtained is supplied with two volumes of ethanol by which the alkali-stable polysaccharides are precipitated.

Purification. The precipitate is collected by centrifugation and redissolved in hot water. This solution is made slightly acid with 2 N HCl. Insoluble material is removed by centrifugation. Ethanol is added to the clear opalescent solution to a final concentration of 45 % (40 ml of ethanol to 50 ml of solution). By adding more ethanol a less pure product is obtained, because then impurities coprecipitate (KCl, phosphates, etc.). If necessary, this purification procedure has to be repeated several times. Finally, the purified polysaccharide is washed twice with ethanol and with ether in the centrifuge tube and subsequently dried in an oven at 70°. The purity of the preparation may be checked by the determination of the glucose content using the anthrone method, or, after hydrolysis of the product, by the method of Luff-Schoorl. The polysaccharides isolated from *Arthrobacter*, strains 1, 29, and 41, had glucose contents of 95 %. The preparations contained about 1 % of nitrogen and approx. 2 % of ash.

The Pflüger method, which was originally used for the isolation of glycogen from liver, has been criticized by several authors. Stetten and Katzen (1961), for example, found that upon action of KOH on glycogen a partial alkaline degradation of this polysaccharide occurred, manifesting itself in a decrease

of the molecular weight. When concentrated alkali acts anaerobically on undegraded glycogen, partial degradation takes place to a polydispers series of relatively stable polysaccharide acids and small amounts of isosaccharinic acid. These degradation products turned out to be identical with the modified glycogen isolated by alkaline methods.

In view of these objections, in a later stage of the investigation the Pflüger method was abandoned and replaced by mechanical procedures, using ultrasonic vibrations or glass beads. When the polysaccharide was isolated by these methods, it is assumed that the isolated product has the same properties as in the intact organism.

5.2. Disintegration of the cells by ultrasonic vibration

The bacterial suspension, cooled by ice, is exposed to ultrasonic vibrations in a MSE ultrasonic disintegrator (Measuring and Scientific Equipment Ltd. London) for half an hour at 20 kc/sec. Subsequently, the resulting suspension is centrifugated in a Servall centrifuge at 20,000 g to remove cell particles and non-disrupted cells. Protein, precipitated by adding trichloroacetic acid (final concentration 2.5% TCA) to the clear bacterial extract, is removed by centrifugation. The clear blue-white opalescent solution is dialysed for 24 h in cellophan against running tap water. The solution is then evaporated in vacuo to one-half the original volume. The polysaccharide is precipitated by adding 2 volumes of ethanol. It is collected by centrifugation, washed with ethanol and ether, and dried in a vacuum desiccator.

Generally, the polysaccharide thus obtained is fairly pure without any subsequent treatment. Further purification can be accomplished by redissolving it in water and reprecipitating it with ethanol.

5.3. Disintegration of the cells in a cell homogeniser

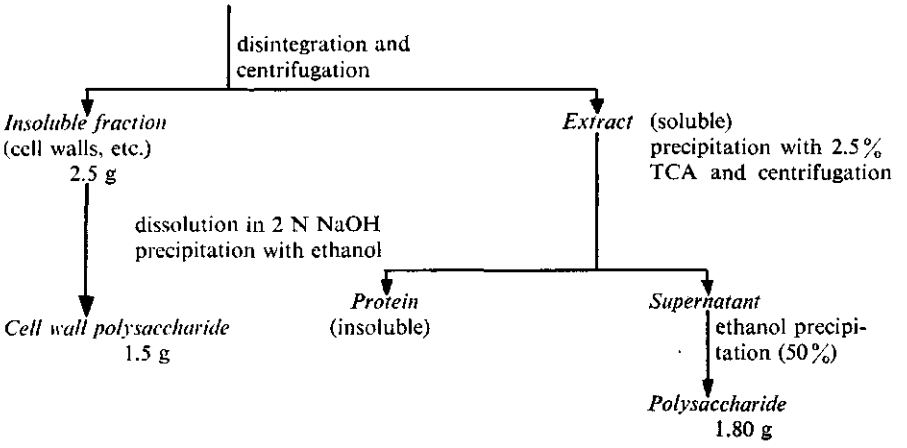
The cells are disintegrated with glass beads (⌀ 0.3 mm) in a cell homogeniser (B. Braun Apparatebau, Melsungen) for 1 min. About 10 g of wet cell material is suspended in 10 ml of water contained in a bottle of 80 ml capacity and then 50 g of glass beads are added. Bottle and contents are previously cooled in ice water and subsequently shaken at a frequency of 4000 c/min. The resulting suspension is then treated as described under 5.2. The disintegration in the homogeniser has the advantage over the procedure described in section 5.2. that more cell material can be treated at one time (10 g of wet cells per bottle as contrasted with 1 g of cells in the MSE disintegrator). Ultrasonic vibration has the additional disadvantage of acting more slowly than disintegration in a Braun homogeniser. With the latter, however, often no complete disruption of all cells is obtained. These non-disrupted cells can eventually be disintegrated by retreating them in the same way.

5.4. Isolation of polysaccharides from *Arthrobacter*

Arthrobacter, strain 1, was cultivated in 2 l of medium (for composition cf. chapter 5, introduction) at 30°. The cells were harvested by centrifugation and washed with distilled water. Dry weight and total carbohydrate were determined in a separate sample. For isolation of the polysaccharides the cells were disintegrated in the Braun homogeniser. Non-disrupted cells and cell fragments were removed by centrifugation in a Servall centrifuge. The latter material was subjected to two further treatments in the Braun homogeniser, until almost no whole cells were present. Cell fragments (cell walls and other insoluble particles) were removed by centrifugation, washed with water (3 times), 96% ethanol (twice) and ether, and finally dried at 100°.

The clear extract was deproteinized by adding trichloroacetic acid (final concentration 2.5%) and the precipitated protein removed by centrifugation. The clear supernatant was supplied with an equal volume of ethanol and the precipitated polysaccharide centrifugated, washed with ethanol (twice) and ether, and dried at 100°. The insoluble fraction was treated with 25 ml of 2 N NaOH for 20 h at room temperature, and subsequently heated at 100° for 20 min. The resulting brown solution was cooled and two volumes of ethanol added. The precipitated cell wall polysaccharide was collected by centrifugation, washed with ethanol and ether, and dried at 100°. The resulting data were as follows:

Washed cells, dry weight 7.85 g, carbohydrate content 3.87 g (as $C_6H_{10}O_5$)



5.4.1. *Chromatographic analysis of the isolated fractions*

a. TCA soluble intracellular polysaccharide. A sample of this polysaccharide (200 mg) was hydrolysed in 10 ml of 1 N sulphuric acid for 16 h at 100°. After removal of the sulphuric acid by precipitation with $Ba(OH)_2$ and concentration of the hydrolysate, the latter was subjected to paper chromatography on Whatman paper no 1. The system n-butanol – acetic acid – water (4:1:5, v/v; upper layer) was used as a solvent; aniline phthalate, dissolved in

n-butanol, was used for detecting the spots. Only one spot with the R_F-value of glucose appeared on the chromatogram.

Glucose was isolated from the hydrolysate in the form of its osazone (m.p. glucosazone 205°). The content of reducing sugar in the hydrolysate was determined according to the method of Luff-Schoorl: 10 mg of polysaccharide gave 10.1 mg of glucose. Thus the polysaccharide was completely built up from glucose.

b. Cell wall polysaccharides. This preparation (100 mg) was hydrolysed in 1 N sulphuric acid for 16 h at 100°. After this treatment it had not completely dissolved. Chromatography of the hydrolysate gave different component sugars. Glucose was found to be the major component sugar; in addition, rhamnose and mannose were present, while two further spots were detected, which have so far not been identified with certainty (presumably galactose and glucosamine).

Determination of reducing sugars in the hydrolysate yielded 6.5 mg (calculated as glucose) from 10.0 mg of polysaccharide.

The total carbohydrate content of *Arthrobacter* can thus be separated into two fractions: a. The intracellular fraction, soluble in TCA, is built up completely from glucose. This polysaccharide was found to have a glycogen-like structure with short average chain length (cf. section 6.5.). This fraction, constituting approximately 50% of the total carbohydrate of the cell, serves as the source of carbon and energy for the endogenous metabolism of the cell (cf. section 5.4.2.). b. A large part of the polysaccharides is found in the insoluble cell fragments. These polysaccharides are built up from more than one component sugar. From the nature of these sugars it was concluded, that this fraction contained the cell wall polysaccharides (SALTON, 1964).

5.4.2. *Nature of the polysaccharides involved in the endogenous metabolism*

Arthrobacter, strain 1, was cultivated in 2 l of the basal salts medium supplied with glucose (composition cf. p. 15); subsequently the cells were harvested by centrifugation and washed with distilled water. The bacteria were resuspended in 1 l of the basal medium without $(NH_4)_2SO_4$ and supplied with glucose (0.5%). After aeration for 5 h at 28°, glucose had disappeared nearly completely from the medium; in this period the carbohydrate content of the cells increased from 23 to 43 per cent (Table 12). At this time 2 g of $(NH_4)_2SO_4$ were added to the culture.

To examine the effect of added nitrogen on the carbohydrous fractions, samples of 400 ml of the culture were taken after 5 and 18.5 h resp. The cells were collected by centrifugation, washed with distilled water and disintegrated in the Braun homogeniser. After removal of the proteins, the soluble polysaccharides were isolated from the extract. On comparing both samples it can be seen, that after the addition of $(NH_4)_2SO_4$ (at 5 h) the dry weight of the cells first decreased slightly and subsequently increased, a phenomenon which was observed several times. The total carbohydrate content of the cells during the 13.5 h period had decreased moderately. The insoluble fraction had increased

TABLE 12. Utilization of the TCA-soluble polysaccharide fraction of nitrogen-deficient cells of *Arthrobacter*, strain 1, on addition of $(NH_4)_2SO_4$.

Time (h)	Dry weight of cells mg/ml of culture	Glucose in medium mg/ml	Cell polysaccharides (mg/ml)			
			Total carbohydrate		TCA-soluble fraction	Insoluble fraction
			mg/ml	%		
0	4.63	4.70	1.06	23		
2	5.24	2.76	1.78	34		
5	6.19	0.30	2.68	43	1.25	1.43
8.5	5.86	0.0	1.98	34		
18.5	6.77	0.0	2.00	30	0.37	1.63

somewhat in contrast with the soluble fraction which had decreased by 0.88 mg per ml of culture.

From these results it is concluded that the soluble polysaccharide fraction is the active endogenous substrate; it is mobilized by adding $(NH_4)_2SO_4$ to serve as the carbon source for the synthesis of new cell material (cf. section 4.3.).

6. DETERMINATION OF THE STRUCTURE OF THE ISOLATED POLYSACCHARIDES

6.1. SPECTRAL INVESTIGATION

6.1.1. *Infrared analysis*

The first indication that the isolated polysaccharides of *Arthrobacter* belong to the glycogen-starch group, came from an infrared analysis of these polysaccharides. The infrared spectra of the polysaccharides of *Arthrobacter*, strains 1, 41, and 159, were recorded with a Perkin-Elmer IR-spectrophotometer; they were compared with the infrared spectra of glycogen and starch, the KBr-technique being used. It was found that the infrared spectra of the bacterial polysaccharides were practically identical with the spectra of glycogen and starch. In the 960–730 cm^{-1} region a number of absorptions occur, characteristic for derivatives of D-glucopyranose (BARKER, et al., 1956). Derivatives with α-configuration (α-glucosidic linkages) absorb at 850 cm^{-1} (type 2a absorption). This peak does not occur in the spectra of derivatives with β-configuration. The latter have an absorption peak at 890 cm^{-1} (type 2b). In addition, α-polyglucosans of the starch type (α-(1→4)-glucosidic linkages) absorb at 930 ± 4 and at 758 ± 2 cm^{-1} (type 1 and 3, respectively), while α-polyglucosans of the dextran type absorb at 917 ± 2 cm^{-1} and at 768 ± 1 cm^{-1}. These data provide a strong indication that the *Arthrobacter* polysaccharides are built up from α(1→4)-linked glucose polymers (Table 13).

TABLE 13. Frequencies (cm^{-1}) of absorption peaks in the 960–730 cm^{-1} region of a number of *Arthrobacter* polysaccharides as compared with those of glycogen and starch.

Absorption type	*Arthrobacter* polysaccharides			Starch	Glycogen
	strain 1	strain 41	strain 159		
1	930	925	930	930	925
2a	850	850	845	850	855
3	760	760	760	760	760

6.1.2. *Spectra of the iodine-polysaccharide complexes*

The spectra of the I_2-polysaccharide complexes were measured in dilute aqueous solution in the region between 400–700 mμ. In figure 9 a number of spectra of iodine-polysaccharide complexes are plotted. The iodine reagent is prepared as follows: citrate buffer (100 ml, pH 6.0; 0.1 M), water (170 ml) and I_2-KI solution (20 ml; 0.2% and 0.4%, resp., w/v) are mixed. The absorption spectrum of the polysaccharide complex (400 μg of polysaccharide in 3 ml of the reagent) is measured in a 1 cm cuvette of the Beckman DU spectrophotometer against an iodine blank. In general, a correlation exists between the branching characteristics and the spectra of the iodine-polysaccharide complexes. When the molecule has a more strongly branched structure, the spectrum of the iodine-polysaccharide complex has its λ_{max} at a shorter wavelength and A_{max} at a lower value.

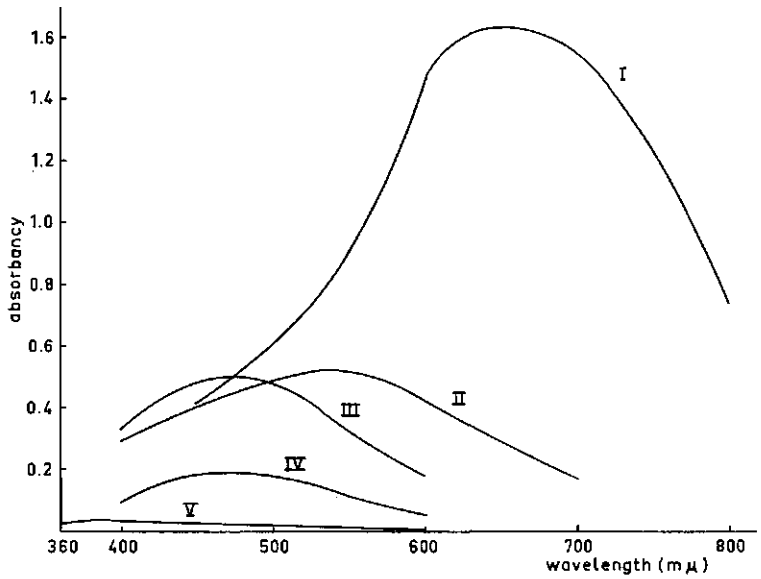


FIGURE 9. Absorption spectra of polysaccharide-iodine complexes: I. amylose; II. amylopectin; III. glycogen of *Agrobacterium tumefaciens*; IV. glycogen of *Sphaerotilus natans*; V. polysaccharide of *Arthrobacter*, strain 1.
Concentration of amylose 200 μg in 3.0 ml I_2-reagent; concentration of the other polysaccharides 400 μg in 3.0 ml I_2-reagent. The spectra were measured in a 1 cm cuvette of a Beckman DU spectrophotometer.

Thus amylose has a maximum at 660 mμ and shows a deep-blue colour with iodine. Amylopectins have λ_{max} at 530–550 mμ and they show a violet colour. Glycogens have a maximum at 420–490 mμ and give a red-brown colour with iodine (ARCHIBALD et al., 1961). The polysaccharide of *Arthrobacter*, strain 1, gave almost no colour with iodine; λ_{max} of the iodine complex was found at 380 mμ. This shows, that these polysaccharides deviate from 'normal' glycogens, which gave a distinct colour with iodine. Glycogens of the latter type have been isolated from *Agrobacterium tumefaciens* and from *Sphaerotilus natans*. Therefore, it may be expected that the *Arthrobacter* polysaccharides have a higher degree of branching.

6.2. END GROUP DETERMINATIONS BY METHYLATION

The molecules of the starch-glycogen class polysaccharides exist of branched-chain molecules, built up from straight chain-fragments of glucose residues linked by α(1→4)-glucosidic bonds. These straight chain-fragments carry branch points, giving rise to side chains via α(1→6)-glucosidic bonds, so that a tree-like structure arises (Figure 10).

Next to the non-reducing end groups, one reducing end group occurs in the molecule, but since the molecule is large, this reducing end group plays an in-

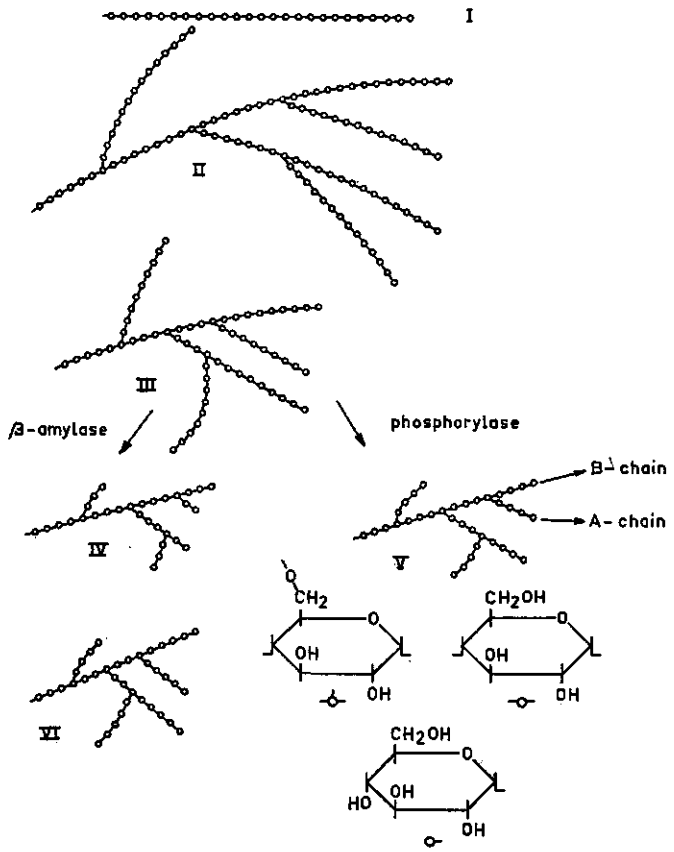


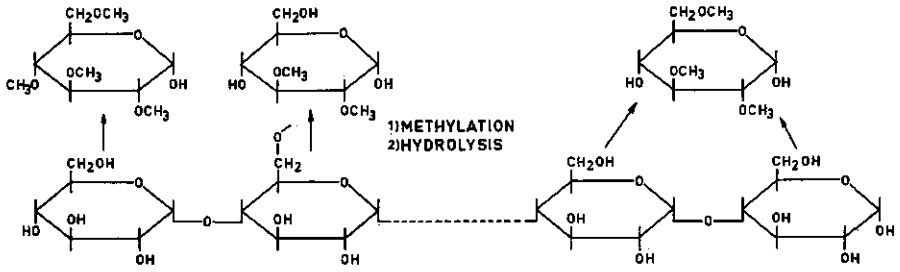
FIGURE 10. Models of polysaccharides of the starch-glycogen class. Only small segments of the molecules are represented. I. amylose, $\overline{CL} = 100$–200;
II. amylopectin (potato), $\overline{CL} = 22$; $\overline{ECL} = 14$ and $\overline{ICL} = 7$.
III. glycogen of *Agrobacterium tumefaciens*, $\overline{CL} = 13$; $\overline{ECL} = 9$ and $\overline{ICL} = 3$.
IV. β-amylase limit dextrin of III. with $\overline{CL} = 6$–7; $\overline{ECL} = 2$–3 and $\overline{ICL} = 3$.
V. phosphorylase limit dextrin of III. with $\overline{CL} = 8$; $\overline{ECL} = 4$ and $\overline{ICL} = 3$.
VI. *Arthrobacter*, strain 1, polysaccharide with $\overline{CL} = 8$; $\overline{ECL} = 5$ and $\overline{ICL} = 2$
—○—, glucose residues in the chain linked by α(1→4)-glucosidic bonds at positions C_1 and C_4.
—○, terminal non-reducing glucose residues.
—○—, glucose residues with α-glucosidic linkages at posititions C_1, C_4 and C_6 (branch points).

significant role only. Furthermore it is to be noticed that when the number of terminal glucose residues in the molecule is n, there are n-2 branch points. Because n is large, the number of branch points and the number of end groups in the molecule are practically equal.

The average chain length $(\overline{CL})$ is defined as the number of glucose residues per mole non-reducing terminal glucose. This value may be found by determination of the number of end groups by methylation or by periodate oxidation.

The outer side chain is the chain fragment from a terminal glucose residue to the first branch point. $\overline{ECL}$ is the mean chain length of the outer side branches; its length may be evaluated by β-amylolysis (cf. section 6.4.1.). The inner chain is the chain fragment between two branch points (average inner chain length $\overline{ICL}$). The total average chain length is composed of the average length of the outer chain + that of the inner chain + branch point: $\overline{CL} = \overline{ICL} + \overline{ECL} + 1$. From this equation $\overline{ICL}$ may be calculated.

The glucose residues in the straight chains, linked by α(1→4)-glucosidic linkages, by methylation and subsequent hydrolysis give rise to 2,3,6,-trimethyl glucose. Non-reducing terminal glucose residues give 2,3,4,6-tetramethyl glucose and glucose residues at the branch points give 2,3-dimethyl glucose. This mixture is separated by chromatography after which the component methylated sugars may be determined separately.



Methylation. (SMITH and MONTGOMERY, 1957). Methylation of the polysaccharides is carried out by the Haworth technique using dimethyl sulphate and sodium hydroxide. The polysaccharide (1 g) is dissolved in approx. 20 ml of water. A mixture of 10 ml of dimethyl sulphate and 30 ml of sodium hydroxide (30%, w/v) is added in 10 equal portions by stirring at 60–70° in the course of 1–2 h. The heating is continued for another half an hour to decompose the excess of dimethyl sulphate. Upon boiling an insoluble white precipitate separates (methylated polysaccharides are insoluble in hot water, more soluble in cold water). The aqueous solution is carefully decanted and the remaining product is dissolved in 20 ml of acetone. Subsequently methylation is carried out once more in acetone solution. After the reaction the acetone is distilled off so that the methylated polysaccharide separates again. Methylation is repeated several times until the methoxyl content of the methylated product does not further increase. Purification is carried out by dissolving the methylated polysaccharide in chloroform, washing the solution with water, drying over anhydric sodium bisulphate and removing the chloroform by distillation. The methylated product may also be obtained by adding light petroleum (b.p. 80–100°) to a dried solution of the product in a small volume of chloroform and by centrifuging the white precipitate. In most instances a white glass-like material was obtained, which was dried at 100°.

Hydrolysis. The methylated polysaccharide (100 mg) together with 5 ml of

water and 0.3 ml of concentrated sulphuric acid are brought into a tube, which is sealed and heated at 100° for 12 h. After the hydrolysis is complete, the tube is opened and the liquid neutralized with barium hydroxide. The precipitated barium sulphate is removed by centrifugation and the clear supernatant concentrated in vacuo. The residue is dissolved in 1 ml of water.

Chromatography. The hydrolysate (20 µl) is brought on to Whatman no 1 paper. Two aliquots are applied: one for detecting the spots on the chromatogram after spraying with aniline-phthalate reagent, and one for the quantitative determination of the methylated sugars after elution of the spots with water. After developing of the chromatogram using n-butanol – ethanol – water (5:1:4) one series of spots are sprayed with aniline-phthalate and subsequently heated for 15 min at 105°.

Five spots appeared on the chromatogram with the following R_{tetra}-values as compared with 2,3,4,6, tetramethyl glucose: 1. 0.38, monomethyl glucose; 2. 0.58, 3,6-dimethyl glucose; 3. 0.64, 2,3-dimethyl glucose; 4. 0.85, 2,3,6-trimethyl glucose; 5. 1.00, 2,3,4,6-tetramethyl glucose. Monomethyl glucose and 3,6-dimethyl glucose arise by partial demethylation of the 2,3,6-trimethyl glucose during hydrolysis (HASSID and ABRAHAM, 1957).

The average chain length of the polysaccharide is found by determination of the amount of tetramethyl glucose in proportion to the total amount of methylated sugar in the hydrolysate: $\overline{CL}$ = (moles of mono + di + tri + tetramethyl glucose) / (moles of tetramethyl glucose).

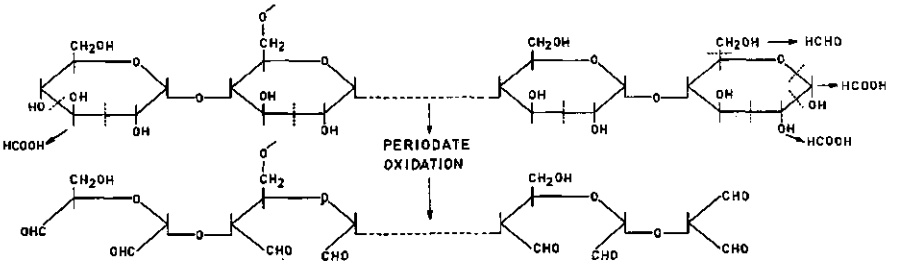
The spots were cut out of the paper and the strips (2 × 2 cm) eluted with 10 ml of water (2 h at 30°). In the resulting solutions the methylated sugars were estimated, using the phenol-sulphuric acid method. The absorbancies were corrected for a paper blank.

The following polysaccharides were investigated: soluble starch, glycogen (NBC) and the polysaccharides from *Arthrobacter*, strains 1 and 29. In these four cases spots with the same R_{tetra}-values were obtained on the chromatogram. From the amounts of tetramethyl glucose found, the average chain lengths were calculated as follows: glycogen (NBC):13; soluble starch: 17; polysaccharide from strain 1: 8; polysaccharide from strain 29: 11.

6.3. END GROUP DETERMINATION BY PERIODATE OXIDATION

Periodate brings about the cleavage of a carbon chain, whenever two or more adjacent hydroxyl groups are present. Upon action of sodium periodate on polysaccharides in aqueous solution, oxidation takes place, resulting in the formation of a polyaldehyde (HASSID and ABRAHAM, 1957). Glucose residues in the straight chain are oxidized at the positions 2 and 3, giving a dialdehyde, but they do not yield formic acid. The same is true of the glucose residues serving as branch points. One mole of sodium periodate is reduced to iodate.

The terminal non-reducing glucose residues consume 2 moles of $NaIO_4$, giving a dialdehyde and liberating 1 mole of formic acid. The terminal reducing glucose residue gives rise to 2 moles of formic acid upon periodate oxidation.



Because in branched polysaccharides of the glycogen-starch class principally non-reducing end groups are present, the amount of formic acid liberated from the reducing terminal unit is so small in comparison with the total amount of formic acid liberated that it can be ignored in the calculation of the percentage end groups. Consequently the method enables the estimation of the number of non-reducing end groups, viz. 1 mole of terminal glucose per mole of formic acid liberated: $\overline{CL}$ = (mg of polysaccharide) / (mmoles of formic acid × 162); 162 = mol. wt. of anhydroglucose.

Procedure. Polysaccharide (40 mg) is dissolved in 2 ml of water, whereupon 2.0 ml of sodium periodate solution (8 g $NaIO_4$ per 100 ml) is added. The solution is kept in the dark at room temperature for 24 h. At the end of the reaction, 0.4 ml of ethylene glycol is added to decompose the excess of periodate. The mixture is kept at room temperature for at least 10 min in order to reduce all the periodate to iodate. The liberated formic acid is then determined by titration with 0.01 N NaOH, bromocresol purple being used as an indicator. A blank is run simultaneously under the same conditions, but here ethylene glycol is added first and $NaIO_4$ afterwards.

In order to follow the velocity of the periodate oxidation reaction, glycogen and amylopectin were oxidized at room temperature. Samples were withdrawn several times in the course of the reaction. The apparent average chain length, which may be calculated from the liberated amount of formic acid was plotted against time (Figure 11). It will be seen that the periodate oxidation was comple-

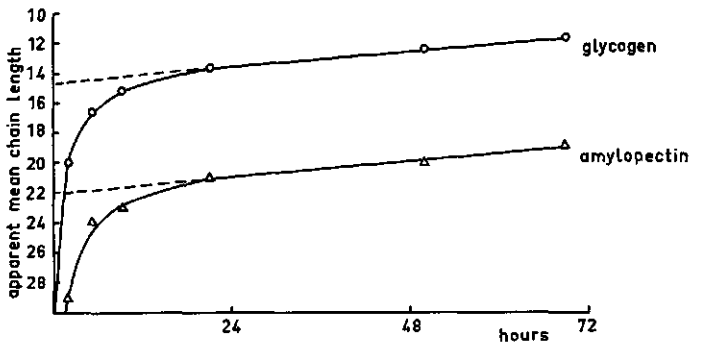


FIGURE 11. Periodate oxidation of amylopectin and glycogen. Apparent mean chain length plotted against time. Extrapolation of the linear part of the curve to zero time gives the exact value of $\overline{CL}$.

ted after approximately 24 h of incubation at room temperature. After that time there was a slight linear production of formic acid with time. This excess of formic acid arose by further oxidation of the dialdehyde groups (over oxidation). Extrapolation of the linear part of the curve to zero time gave the exact value of $\overline{CL}$.

6.4. Enzymatic procedures for the analysis of polysaccharides of the glycogen-starch group

6.4.1. *Action of* β-*amylase* (α-1,4-glucan maltohydrolase, EC 3.2.1.2.)

When β-amylase acts on branched polysaccharides of the glycogen-starch group, the outer chains are broken down giving maltose. This reaction proceeds purely hydrolytic, only α(1→4)-glucosidic bonds being ruptured. The action starts from the non-reducing end of the outer side chains of the molecule, attacking alternate glucosidic bonds, continuing until further enzyme action is blocked by a branch point in the molecule. After exhaustive treatment of the branched polysaccharides with β-amylase a high-molecular-weight 'limit dextrin' remains. This β-limit dextrin has all the branches of the original substrate molecule, but the outer chains are reduced in length to stubs of only 2 or 3 glucose residues (Peat, Whelan and Thomas, 1952; Summer and French, 1956).

Procedure for β-amylolysis (Kjølberg and Manners, 1962). Polysaccharide (10 mg) is dissolved in 1 ml of water; 2 ml of 0.2 M acetate buffer, pH 4.6, and 0.25 ml of β-amylase (cryst. β-amylase from barley, 5 mg/ml) are added, and the solution is made up with water to 10 ml. This mixture is incubated at 30° for 24 h and then maltose is determined in a 0.2 ml sample by the method of Somogyi-Nelson.

6.4.2. *Action of* α-*amylase* (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1.)

α-Amylase also hydrolyses exclusively α(1→4)-glucosidic linkages in the glycogen or amylopectin molecule. In contrast with β-amylase, α-amylase acts randomly on the α(1→4)-linkages, though the α(1→6) bonds constituting the branch points of amylopectin and glycogen remain unattacked. Not only linkages in the outer chains are broken, but also α(1→4)-glucosidic linkages between two branch points are hydrolysed. Amylopectin and glycogen are thus broken down by low concentrations of human salivary α-amylase to yield maltose (42%), maltotriose (28%) and low molecular weight limit dextrins (Walker and Whelan, 1960).

Procedure for α-amylolysis. Polysaccharide (10 mg), NaCl (1.25 ml; 0.5%), citrate buffer (1.25 ml, 0.1 M; pH 6.2), and α-amylase (0.25 ml; 5 mg cryst. enzyme/ml) are mixed and water added to 10 ml. The mixture is incubated at 30° for 24 h. Reducing sugars are then determined in 0.2 ml portions by the method of Somogyi-Nelson, using maltose as the standard.

6.5. PROPERTIES OF THE ISOLATED POLYSACCHARIDES FROM *ARTHROBACTER*

The properties of the isolated polysaccharides of *Arthrobacter* (Table 14) have been compared with those of glycogens from several other bacterial and animal species (Table 15 and Figure 10).

TABLE 14. Properties of the isolated *Arthrobacter* polysaccharides

Arthrobacter strain	$\overline{CL}$	β-amylolysis limit (%)	$\overline{ECL}$	$\overline{ICL}$	λ_{max} of I_2-complex
1	7–8	31	5	1–2	380 mμ
4	9	37	6	2	
8	8	28	5	2	
11	8	23	4	3	
29	9	28	5	3	
41	8	25	4–5	2–3	
159	8–9	29	5	2–3	
166	7–8	24	4	2–3	

$\overline{CL}$ = average chain length as determined by periodate oxidation
$\overline{ECL}$ = average exterior chain length being the number of glucose residues removed by β-amylase + 2.5
$\overline{ICL}$ = average interior chain length = $\overline{CL} - \overline{ECL} - 1$
β-amylolysis limit (%) = percentage of polysaccharide released as maltose by the action of β-amylase

TABLE 15. Properties of glycogens from several other bacterial and animal species and of plant amylopectins.

Organism	$\overline{CL}$	β-amylolysis limit (%)	$\overline{ECL}$	$\overline{ICL}$	λ_{max} of I_2-complex (mμ)
Agrobacterium tumefaciens	13	52	9	3	470
Sphaerotilus natans	13	57	10	2	480
Saccharomyces cerevisiae[1]	12	50	8–9	2–3	brown
Bacillus megaterium[2]	9	45	6–7	1–2	light brown
Escherichia coli[3]	12	56	9	2–3	440
Aerobacter aerogenes[3]	13	53	9	3	485
Tetrahymena pyriformis[4]	13	44	8	4	
Cat (liver)[5]	13		9–10	2–3	465
Cock (liver)[5]	13		7–8	4–5	440
Helix pomatia[5]	7		4	2	425
Human liver[5]	14		9	4	460
Oyster[5]	10		6–7	2–3	440
Horse muscle[5]	11		7	3	460
Rabbit liver[5]	13		5–6	6–7	455
Rabbit muscle[5]	12		8	3	490
Potato amylopectin[5]	23		14–15	7–8	540
Protozoal amylopectin[5]	22		15–16	5–6	530
Waxy maize starch[5]	22		14–15	6–7	530

[1]NORTHCOTE (1953); [2]BARRY et al. (1952); [3]SIGAL et al. (1964); [4]MANNERS and RYLEY (1952); [5]ARCHIBALD et al. (1961).

The results recorded in table 14 show a close resemblance between the polysaccharides of different *Arthrobacter* strains. Average chain lengths ($\overline{CL}$) varied between 7 and 9, β-amylolysis limits between 23 and 37 per cent; $\overline{ECL}$ between 4 and 5, and $\overline{ICL}$ between 2 and 3. Upon action of α-amylase on the *Arthrobacter* polysaccharides 30–45 per cent reducing sugar (as maltose) was set free (α-amylolysis limit). Iodine gave only a very slight yellow-brown colour. The *Arthrobacter* polysaccharides have shorter chain lenghts than glycogens from some other microorganisms (Table 15). The glycogen of *Agrobacterium tumefaciens* was isolated from a strain present in the collection of the Laboratory of Microbiology at Wageningen. The glycogen of *Sphaerotilus natans* was isolated by VAN VEEN. The properties of the glycogens of the other organisms in table 15 are derived from the literature.

MANNERS and collaborators have examined a large number of animal glycogens and amylopectins of plants (ARCHIBALD et al., 1961). From the data of table 15 it appears that most glycogens have an average chain length of 10–14, except those of *Bacillus megaterium* and *Helix pomatia*, which are shorter. In most cases the outer chains are two or three times longer than the inner chains.

The polysaccharides of the glycogen-starch class in addition to amylopectins, amylose and glycogens include the glycogen phosphorylase limit dextrins (KJØLBERG and MANNERS, 1962; cf. also Table 16). The phosphorylase limit dextrins arise on incubation in vitro of glycogens with phosphorylase until no

TABLE 16. Classification of the polysaccharides of the glycogen-starch class according to KJØLBERG and MANNERS (1962).

Polysaccharide	$\overline{CL}$	β-amylolysis limit (%)	$\overline{ECL}$	$\overline{ICL}$	α-amylolysis limit (%)	I_2-complex λ_{max} (mμ)
Amylopectins	18–24	50–60	9–16	6–8	85–92	530–550
Glycogens	10–14	40–50	6–10	2–4	75–85	420–500
Glycogen phosphorylase limit dextrins	6–8	14–28	2–4	2–4	33–48	–
Arthrobacter polysaccharides	7–9	23–37	4–5	2–3	30–45	–

further action occurs (cf. section 7.5.); their properties resemble those of the isolated *Arthrobacter* polysaccharides. They have also been found in vivo by CALDERBANK et al. (1960) in the liver of a patient suffering from glycogen storage disease (type III). This compound had a $\overline{CL} = 6$ and a β-amylolysis limit of 14%. The abnormal structure was explained by a deficiency of the enzyme amylo-(1→6)-glucosidase in the liver as a result of which glycogen could only be broken down to the phosphorylase limit dextrin stage, which accumulated in the liver.

Another case of phosphorylase limit dextrins was encountered in a study of the yeast glycogen (EATON, 1963). He found that in yeast two glycogen components are serving as endogenous substrates (cf. section 4.1.). The glycogen pool was isolated from the cells and it was separated into a heavy and a light fraction

in the ultracentrifuge. The average numbers of glucose residues external to the α(1→6)-glucosidic linkages ($\overline{ECL}$) of the molecules of both fractions were estimated by phosphorylase or β-amylase degradation. The external chains of the heavy fraction were found to be considerably longer than those of the light fraction ($\overline{ECL}$ of the heavy fraction 9.4 and of the light fraction 4.2 glucose residues). These results suggested that the two glycogen components, defined by kinetic analysis, consist of a. chains of glucose external to α(1→6)-glucosidic linkages which is the rapidly metabolized component and b. the remainder of the glycogen molecule which is the more slowly metabolized component. From these results EATON concluded that the velocity by which glycogen is degraded in yeast depends on the activity of the α(1→6)-glucosidase (debranching enzyme). Whereas the α(1→4)-glucosidic linkages in the outer chains are rapidly broken down by phosphorylase, the α(1→6)-glucosidic linkages are only slowly hydrolysed.

From a kinetic study of substrate disappearance in *Arthrobacter* (section 4.1.) it was concluded that in this organism only one glycogen component is present, which is only slowly broken down. This glycogen ($\overline{ECL}$ = 4–5) thus corresponds to the light glycogen fraction in yeast, suggesting that glycogen degradation in *Arthrobacter* is also limited by a slow action of the α(1→6)-glucosidase.

After termination of this investigation a short communication appeared on the isolation and characterization of the glycogen from *Arthrobacter* sp. NRRL B1973 (GHOSH and PREISS, 1965). They found the following properties of the isolated product: $\overline{CL} = 9$; $\overline{ECL} = 6$; $\overline{ICL} = 2$; α-amylolysis limit 54%; β-amylolysis limit 34% and λ_{max} of the I_2-complex between 420–440 mμ, being in full agreement with the properties of the products of the present investigation.

7. BIOSYNTHESIS AND BREAKDOWN OF THE INTRACELLULAR POLYSACCHARIDE OF *ARTHROBACTER*

Synthesis of polysaccharides occurs by transfer of glucosyl groups from a glucosyl donor to an acceptor according to the general equation

$$R\text{—}O\text{—}X + HOR' \leftrightarrows R\text{—}OR' + HOX$$

In this equation ROX functions as the glucosyl donor. Examples of such a donor are: disaccharides, polysaccharides, sugar phosphates and sugar nucleotides. Since the reaction proceeds by transfer of the glucosyl group (sugar residue without hemiacetal oxygen) instead of the glucoside group, the term 'transglucosylase' for the enzyme and 'transglucosylation' for the reaction mechanism were introduced (HEHRE, 1951).

The polysaccharide acceptor (HOR') must be present during the reaction in low concentrations to act as a 'primer'. Several bacteria can synthesize polysaccharides from disaccharides. For example, sucrose may function as glucosyl or as fructosyl donor in the formation of dextran and levan, respectively. Dextransucrase (*Leuconostoc mesenteroides* and *Leuconostoc dextranicus*) is an extracellular enzyme, forming dextran from sucrose (HEHRE, 1946).

$$\underset{\text{sucrose}}{\text{n } \alpha\text{-D-glucosyl-}\beta\text{-D-fructoside}} \rightarrow \underset{\text{polysaccharide}}{\alpha(1\rightarrow6)\text{-D-glucose}_n} + \text{n D-fructose}$$

Neisseria perflava forms a glycogen-like polysaccharide from sucrose by the action of amylosucrase (HEHRE, HAMILTON and CARLSON, 1949).

$$\underset{\text{sucrose}}{\text{n } \alpha\text{-D-glucosyl-}\beta\text{-D-fructoside}} \rightarrow \underset{\text{polysaccharide}}{\alpha(1\rightarrow4)\text{-D-glucose}_n} + \text{n D-fructose}$$

Mutants of *E.coli* transfer maltose into a starch-like polysaccharide and glucose under the influence of amylomaltase (MONOD and TORRIANI, 1948).

$$\underset{\text{maltose}}{\text{n } \alpha\text{-D-glucosyl-}\alpha\text{-D-glucose}} \rightarrow \underset{\text{polysaccharide}}{\alpha(1\rightarrow4)\text{-D-glucose}_n} + \text{n D-glucose}$$

Starch and glycogen phosphorylase. In 1937 CORI et al. showed that glycogen was converted to α-D-glucose-1-phosphate in the presence of inorganic phosphate and an enzyme, phosphorylase, present in muscle extracts. It soon appeared that the reaction was freely reversible:

$$x\ \alpha\text{-D-glucose-1-phosphate} + (\text{D-glucose})_n \leftrightarrows (\text{D-glucose})_{n+x} + x\ \text{phosphate}$$

This reaction takes place by transfer of a glucosyl group from glucose-1-phosphate to a terminal non-reducing glucose residue of the acceptor polysaccharide, forming long linear chains joined through α(1→4) linkages. The equilibrium of the phosphorylase reaction is not affected by the concentration of the polysaccharide, provided a certain minimum concentration is exceeded, because the concentration of the non-reducing terminal D-glucose units does not change on the addition of new D-glucose units. On the other hand, the equilibrium varies

with the pH; at equilibrium the ratio of inorganic phosphate to α-D-glucose-1-phosphate at pH 7.0 amounts to 3.

CORI and CORI (1943) succeeded in obtaining the biosynthesis of glycogen in vitro by the simultaneous action of phosphorylase and branching enzyme. Phosphorylase led to elongation of the glycogen molecules by attaching glucose residues via α(1→4)-glucosidic linkages until a certain chain length was reached. Afterwards the branching enzyme acted on these straight chains forming the branched structure of glycogen. At first it was thought that glycogen synthesis in vivo also proceeded under the influence of the enzyme phosphorylase. Later it appeared, that in vivo phosphorylase functions primarily in the degradation of glycogen. This was concluded from the following fact. The equilibrium of the phosphorylase reaction is reached when the ratio of inorganic phosphate to glucose-1-phosphate is approximately 3 at pH 7; synthesis only takes place when this ratio is lower than 3. In animal tissues this ratio is always much higher than 3 (values up to 300 were observed) and simular values were obtained in the present investigation with yeast and *Arthrobacter* cells. With such high intracellular concentrations, glycogen synthesis can not proceed by this reaction (LELOIR and CARDINI, 1962).

Glycogen synthetase. An enzyme different from phosphorylase catalysing the synthesis of glycogen was first detected in liver and subsequently in many other animal tissues and in yeast. This enzyme uses UDPG (uridine diphosphate glucose) as the substrate (LELOIR and CARDINI, 1962).

$$x\ \text{UDP-D-glucose} + \alpha(1\rightarrow4)\text{-D-glucose}_n \rightarrow \alpha(1\rightarrow4)\text{-D-glucose}_{n+x} + x\ \text{UDP}$$

The reaction is independent of the phosphate concentration. The equilibrium constant of this reaction was found to be about 250, so that the conversion of the nucleotide-bound glucose to glycogen is nearly quantitative.

The synthesis of starch and glycogen in vivo proceeds as follows: the attachment of glucose residues to the acceptor polysaccharide proceeds by UDPG as the substrate. In this way glucose polymers arise in which the glucose residues are bound in straight chains by α(1→4) linkages. When these straight chains reach a length sufficient to become a substrate for the branching enzyme, a branch point is formed by rupture of an α(1→4) linkage in the straight chain and transfer of the outer segment of the chain to the carbon atom 6 of a nonterminal glucose unit. In this way a branch point is introduced into the molecule by means of an α(1→6)-glucosidic bond. The chains are then again elongated whereupon the action of the branching enzyme is repeated.

Synthesis and breakdown of these polysaccharides thus proceed by different pathways, degradation taking place by the system phosphorylase and debranching enzyme. Phosphorylase degrades the polysaccharide beginning at the non-reducing end groups of the molecule, each time removing a glucose residue as glucose-1-phosphate. Exclusively α(1→4)-glucosidic bonds are ruptured by phosphorylase. Its action ceases when the first tier of branch points are approached; the remaining part of the molecule is called a phosphorylase limit dextrin. Before phosphorylase may act further on this limit dextrin, the branch points (α(1→6)-

plete system without glucose. min

7.3. POLYSACCHARIDE SYNTHETASE IN *ARTHROBACTER*, STRAIN 1

Systematic name: UDPglucose:α-1,4-glucan α-4-glucosyltransferase, EC 2.4.1.11.

Enzyme assay. Two methods may be employed for the assay of the polysaccharide synthetase activity, viz.

glucosidic linkages) have to be broken down by a debranching enzyme (amylo-(1→6)-glucosidase in liver or R-enzyme in plants). In this way new outer chains arise, which on their turn may be degraded by the action of phosphorylase (CORI and LARNER, 1951).

560 mμ. The addition of 0.1 ml of 0.1 N HCl (10 μM H^+) instead of the bacterial extract gave a decrease of the absorbancy at 560 mμ of 0.37. The incubation was carried out at 20°. One unit of enzyme is defined as the amount of enzyme, catalysing the conversion of 1 μM glucose per min under the above-mentioned conditions. The specific activity is expressed as units per mg of protein. The protein concentration of the *Arthrobacter* extract was 10 mg per ml. The specific activity of this extract was calculated from figure 12 and amounted to 0.4 U per mg of protein at 20°.

7.2. PHOSPHOGLUCOMUTASE IN EXTRACTS OF ARTHROBACTER, STRAIN 1

a. determination of the UDP formed from UDPG,
b. determination of the incorporation of radioactive glucose from UDPG-C^{14} into the polysaccharide.

The former method was applied in the present investigation; UDP was estimated by means of pyruvate kinase: UDP + PEP $\leftrightarrows$ UTP + pyruvic acid. Phosphoenolpyruvate (PEP) transfers the phosphate group to UDP by the catalysing action of pyruvate kinase. The pyruvate liberated is converted into the dinitrophenylhydrazone and estimated colorimetrically by measuring the absorbancy at 520 mμ (LELOIR and GOLDEMBERG, 1962).

Reagents: a. Polysaccharide solution, 40 mg/ml; b. glycine buffer, pH 8.3, 0.75 M containing 0.025 M EDTA; c. 0.05 M glucose-6-phosphate, pH 7.0 (sodium salt); polysaccharide, buffer and glucose-6-phosphate solutions are mixed in equal volumes. d. UDPG, sodium salt, 0.00377 M; e. pyruvate kinase, diluted to 2 mg protein per ml in 0.1 M $MgSO_4$; f. 0.01 M PEP (sodium salt) dissolved in 0.4 M KCl; g. 0.1% dinitrophenylhydrazine in 2 N HCl; h. standard UDP solution, 0.01 M.

The polysaccharide – buffer – glucose-6-phosphate mixture (0.1 ml), UDPG solution (0.1 ml) and bacterial extract (0.1 ml) are incubated at 30°. The reaction is started on the addition of the UDPG solution. The reaction is stopped after different periods of time by heating the tubes for 1 min in a boiling-water bath. The UDP formed is determined by adding 0.05 ml PEP and 0.025 ml of pyruvate kinase, the tubes incubated for 15 min at 30° and then the pyruvate measured by adding 0.3 ml dinitrophenylhydrazine. After 5 min 0.4 ml 10 N NaOH and 2.2 ml ethanol are added. The brown solution is centrifuged and the absorbancy of the supernatant is measured at 520 mμ against a blank. The standard contained 0.1 μM UDP per tube. The activity of the crude extract (5.0 mg protein per ml) was rather low. In 90 min 0.06 μM UDP was formed with 0.1 ml of extract at 30°. Specific activity amounted to 0.0014 U per mg protein (Fig. 15). This low activity may have been due to the fact that the polysaccharide synthetase of *Arthrobacter* accepts as the substrate an NDPG compound other than UDPG. PREISS et al. (1964) and GREENBERG and PREISS (1964) found two different enzyme systems for the synthesis of glucose polymers in cell-free extracts of *Arthrobacter* sp. NRRL B1973. One system, sedimenting at 30,000 g, incorporated glucose from UDP-glucose with a transglucosylase activity of 0.02 U. The second system was present in the 105,000 g supernatant. It incorporated glucose-C^{14} from ADP-glucose-C^{14} into a product that was insoluble in 80 per

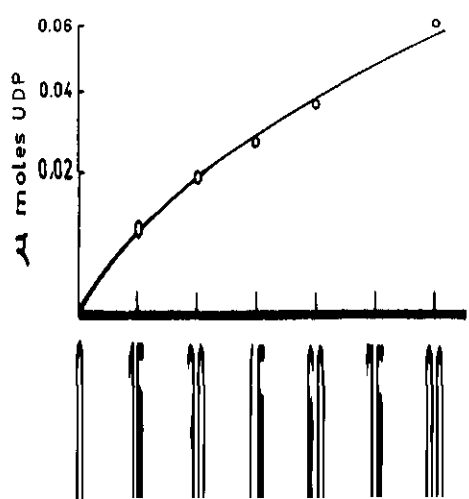


FIGURE 15. Polysaccharide synthetase (UDPG-polysaccharide transglucosylase) activity of cell-free extracts of *Arthrobacter*, strain 1.
UDPG (0.377 μmoles), *Arthrobacter* polysaccharide (1.3 mg); glycine buffer, pH 8.3 (25 μmoles), EDTA (1 μmole), glucose-6-phosphate (1.6 μmole) and bacterial protein (1 mg) were incubated

with the pH; at equilibrium the ratio of inorganic phosphate to α-D-glucose-1-phosphate at pH 7.0 amounts to 3.

CORI and CORI (1943) succeeded in obtaining the biosynthesis of glycogen in vitro by the simultaneous action of phosphorylase and branching enzyme. Phosphorylase led to elongation of the glycogen molecules by attaching glucose residues via α(1→4)-glucosidic linkages until a certain chain length was reached. Afterwards the branching enzyme acted on these straight chains forming the branched structure of glycogen. At first it was thought that glycogen synthesis in vivo also proceeded under the influence of the enzyme phosphorylase. Later it appeared, that in vivo phosphorylase functions primarily in the degradation of glycogen. This was concluded from the following fact. The equilibrium of the phosphorylase reaction is reached when the ratio of inorganic phosphate to glucose-1-phosphate is approximately 3 at pH 7; synthesis only takes place when this ratio is lower than 3. In animal tissues this ratio is always much higher than 3 (values up to 300 were observed) and simular values were obtained in the present investigation with yeast and *Arthrobacter* cells. With such high intracellular concentrations, glycogen synthesis can not proceed by this reaction (LELOIR and CARDINI, 1962).

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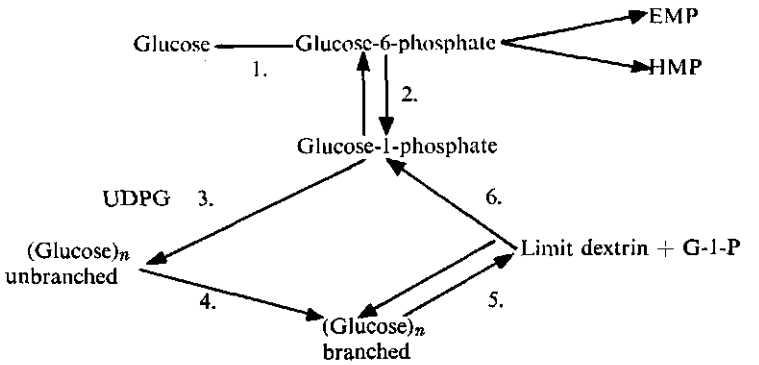
glucosidic linkages) have to be broken down by a debranching enzyme (amylo-(1→6)-glucosidase in liver or R-enzyme in plants). In this way new outer chains arise, which on their turn may be degraded by the action of phosphorylase (CORI and LARNER, 1951).

Scheme for the biosynthesis and breakdown of polysaccharides of the glycogen-starch type.



1. Hexokinase: Glucose + ATP→Glucose-6-phosphate + ADP + H^+
2. Phosphoglucomutase: Glucose-1-phosphate $\rightleftarrows$ Glucose-6-phosphate
3. Glycogen synthetase (UDPG-glycogen transglucosylase):
   $(Glucose)_n + UDPG \rightarrow (Glucose)_{n+1} + UDP$
4. Branching enzyme $(Glucose)_n$, unbranched → $(Glucose)_n$, branched
5. Phosphorylase: $(Glucose)_n + P_i \rightleftarrows (Glucose)_{n-1}$ + Glucose-1-phosphate
6. Debranching enzyme (α(1→6)-glucosidase): hydrolysis of α(1→6) branch points.

Investigations by MORRIS (1960) and by ZAGALLO and WANG (1962) have shown that the glucose metabolism of *Arthrobacter globiformis* proceeds mainly by two different pathways: the Embden-Meyerhof (EMP) and the hexose monophosphate (HMP) pathways. The above-mentioned authors found about 65% utilization of the glucose by way of the EMP pathway and 35% by way of the HMP system.

The conversion of glucose-1-phosphate into UDPG proceeds by the action of UDPG pyrophosphorylase: G-1-P + UDP$\rightleftarrows$UDPG + PP. Hereafter incorporation of the glucose residues into the polysaccharide takes place. The conversion of 1 mole of glucose into the glucose polymer thus requires two moles of ATP. The polysaccharide is laid down in the cell in a branched form. This is the most suitable form for the accumulation of a large number of glucose residues in the cell, since owing to the high molecular weight of the polysaccharide the osmotic pressure remains low. In addition, highly branched polysaccharides have a relatively low viscosity as contrasted with the unbranched or poorly branched structures of amylose and of amylopectins, which are very viscous.

On degradation of the polysaccharides, glucose appears in the phosphorylated form as glucose-1-phosphate and may be utilized either for endogenous respiration or for the synthesis of new cell components.

The enzyme systems for the synthesis and breakdown of the glycogen-like polysaccharide of *Arthrobacter* have been studied in the present investigation.

Special attention was given to the properties of the branching and debranching enzymes. It may be assumed that a close relationship exists between the properties of these enzymes and the branching characteristics of the polysaccharides formed. So far these enzymes have been studied in bacteria only sporadically (ZEVENHUIZEN, 1964; SIGAL et al., 1965).

7.1. HEXOKINASE IN EXTRACTS OF *ARTHROBACTER*, STRAIN 1

Systematic name: ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1.

Preparation of the bacterial extract. *Arthrobacter*, strain 1, was cultivated in the basal salts medium with glucose as the sole source of carbon (composition, cf. section 3.5; 0.3% $(NH_4)_2SO_4$). The cells were harvested at the end of the exponential growth phase. They were disintegrated in a cell homogeniser (Braun Apparatebau, Melsungen) for 1 min using glass beads. Cell fragments were removed by centrifugation in a Servall centrifuge at 20,000 g. Finally, the clear extract was dialysed for 20 h against running tap water in cellophan. The protein content in the extract was estimated according to LOWREY et al. (1951). Hexokinase was assayed by measuring the acid liberated during the reaction:

$$ATP + \text{hexose} \rightarrow ADP + \text{hexose-6-phosphate} + H^+$$

This was done colorimetrically in the presence of an acid-base indicator (cresol red) and a buffer (glycyl-glycine - NaOH) (DARROW and COLOWICK, 1962). In this system the decrease of the extinction at 560 mμ is proportional to the amount of the liberated acid.

Reagents. a. 0.006% cresol red; 1.6% $MgCl_2 \cdot 6H_2O$; b. 0.1 M ATP (di Na-salt); c. 0.1 M NaOH; d. 0.1 M glycyl-glycine - NaOH buffer, pH 9.0; e. 0.2 M glucose. Reagent a. (7 ml) was mixed with reagent b. (1.5 ml), and neutralized with 0.1 M NaOH until the indicator became reddish purple, 3 ml of reagent d. were added and the mixture diluted with distilled water to 30 ml.

The following solutions were incubated in a 1 cm cuvette (Beckman): 2.5 ml of the above assay solution, 0.4 ml 0.2 M glucose and 0.1 ml bacterial extract. The reaction was measured by determination of the change in absorbancy at

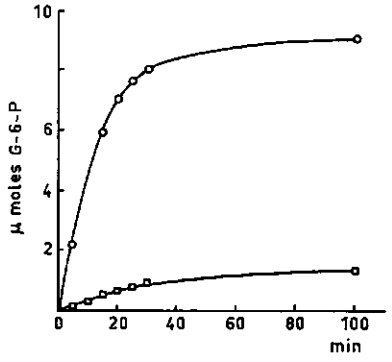


FIGURE 12. Hexokinase in extracts of *Arthrobacter*, strain 1. ○, complete system; □, complete system without glucose.

560 mμ. The addition of 0.1 ml of 0.1 N HCl (10 μM H^+) instead of the bacterial extract gave a decrease of the absorbancy at 560 mμ of 0.37. The incubation was carried out at 20°. One unit of enzyme is defined as the amount of enzyme, catalysing the conversion of 1 μM glucose per min under the above-mentioned conditions. The specific activity is expressed as units per mg of protein. The protein concentration of the *Arthrobacter* extract was 10 mg per ml. The specific activity of this extract was calculated from figure 12 and amounted to 0.4 U per mg of protein at 20°.

7.2. PHOSPHOGLUCOMUTASE IN EXTRACTS OF *ARTHROBACTER*, STRAIN 1

Systematic name: D-glucose-1,6-diphosphate:D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1.

Reagents: cysteine 0.1 M; $MgSO_4$ 6 × 10^{-3} M; glucose-1-phosphate 0.02 M; bacterial extract.

Enzyme assay (NAJAR, 1955). Of each reagent 0.1 ml is pipetted in a tube and placed in a water bath at 30°. The enzyme activity can be measured in two manners.

a. By measuring the disappearance of acid-labile phosphate (glucose-1-phosphate). After incubation, the reaction is stopped by adding 1 ml of 5 N H_2SO_4 and 3.7 ml of water. The contents of the tube are heated for 3 min at 100°, to hydrolyse the remaining glucose-1-phosphate. Under these conditions glucose-6-phosphate is stable. The liberated inorganic phosphate is determined by the method of Fiske-Subbarow.

b. By measuring the glucose-6-phosphate formed. In contrast with glucose-1-phosphate, glucose-6-phosphate has reducing properties. It can thus be determined by the method of Somogyi-Nelson.

$MgSO_4$ appeared to have a stimulating effect on the phosphoglucomutase activity of a crude dialysed extract of *Arthrobacter*, strain 1. The specific activity of the extract amounted to approximately 0.05 U per mg protein at 30° (Fig. 13). Optimum pH of the phosphoglucomutase activity was pH 8.0. At equilibrium about 90 to 95% glucose-1-phosphate had been converted into glucose-6-phosphate.

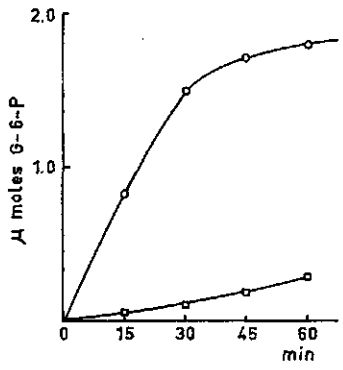


FIGURE 13. Phosphoglucomutase in extracts of *Arthrobacter*, strain 1. ○, complete system containing 10 μmoles cysteine, 2 μmoles glucose-1-phosphate, 0.6 μmoles $MgSO_4$ and 1 mg bacterial protein in a total volume of 0.4 ml.
□, complete system plus 100 μmoles NaF.

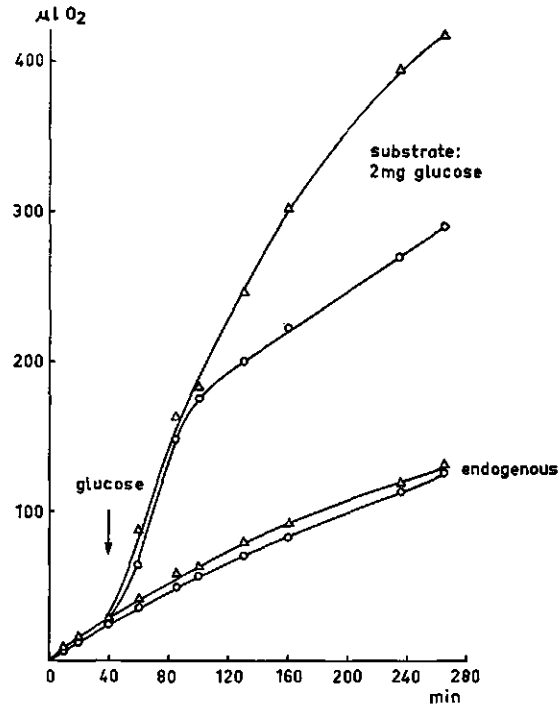


FIGURE 14. Oxygen uptake of a suspension of *Arthrobacter*, strain 1, (1 ml) incubated with 2 mg glucose, in the absence of NaF (circles) and in the presence of 100 µmoles NaF (triangles). Lower pair of curves: endogenous respiration.

NaF has a strong inhibiting effect on the phosphoglucomutase activity (Fig. 13). Since phosphoglucomutase is part of the enzyme system catalysing the synthesis of polysaccharides, it might be expected that NaF has an inhibiting effect on this synthesis. This was clearly shown in a Warburg experiment with a washed suspension of *Arthrobacter*, strain 1. One ml of this suspension containing 3.59 mg of cells (dry weight) was pipetted into Warburg vessels, and 2.0 mg of glucose was added as the substrate. The oxygen consumption of this suspension was measured in the presence and in the absence of NaF (Fig. 14). It was found that the respiration rate was not affected by NaF. In the vessel without NaF glucose had disappeared 60 min after its addition. When the cell suspension was analysed approximately 50% of the added glucose was found to be assimilated in the form of polysaccharides (cf. section 4.1.). In the vessel with NaF the respiration of the free glucose continued for a much longer period of time, demonstrating that polysaccharide synthesis proceeded at a much lower rate. Even after 4 h free glucose was still present in the medium.

7.3. POLYSACCHARIDE SYNTHETASE IN *ARTHROBACTER*, STRAIN 1

Systematic name: UDPglucose:α-1,4-glucan α-4-glucosyltransferase, EC 2.4.1.11.

Enzyme assay. Two methods may be employed for the assay of the polysaccharide synthetase activity, viz.

a. determination of the UDP formed from UDPG,
b. determination of the incorporation of radioactive glucose from UDPG-C^{14} into the polysaccharide.

The former method was applied in the present investigation; UDP was estimated by means of pyruvate kinase: UDP + PEP $\leftrightarrows$ UTP + pyruvic acid. Phosphoenolpyruvate (PEP) transfers the phosphate group to UDP by the catalysing action of pyruvate kinase. The pyruvate liberated is converted into the dinitrophenylhydrazone and estimated colorimetrically by measuring the absorbancy at 520 mμ (LELOIR and GOLDEMBERG, 1962).

Reagents: a. Polysaccharide solution, 40 mg/ml; b. glycine buffer, pH 8.3, 0.75 M containing 0.025 M EDTA; c. 0.05 M glucose-6-phosphate, pH 7.0 (sodium salt); polysaccharide, buffer and glucose-6-phosphate solutions are mixed in equal volumes. d. UDPG, sodium salt, 0.00377 M; e. pyruvate kinase, diluted to 2 mg protein per ml in 0.1 M $MgSO_4$; f. 0.01 M PEP (sodium salt) dissolved in 0.4 M KCl; g. 0.1% dinitrophenylhydrazine in 2 N HCl; h. standard UDP solution, 0.01 M.

The polysaccharide – buffer – glucose-6-phosphate mixture (0.1 ml), UDPG solution (0.1 ml) and bacterial extract (0.1 ml) are incubated at 30°. The reaction is started on the addition of the UDPG solution. The reaction is stopped after different periods of time by heating the tubes for 1 min in a boiling-water bath. The UDP formed is determined by adding 0.05 ml PEP and 0.025 ml of pyruvate kinase, the tubes incubated for 15 min at 30° and then the pyruvate measured by adding 0.3 ml dinitrophenylhydrazine. After 5 min 0.4 ml 10 N NaOH and 2.2 ml ethanol are added. The brown solution is centrifuged and the absorbancy of the supernatant is measured at 520 mμ against a blank. The standard contained 0.1 μM UDP per tube. The activity of the crude extract (5.0 mg protein per ml) was rather low. In 90 min 0.06 μM UDP was formed with 0.1 ml of extract at 30°. Specific activity amounted to 0.0014 U per mg protein (Fig. 15). This low activity may have been due to the fact that the polysaccharide synthetase of *Arthrobacter* accepts as the substrate an NDPG compound other than UDPG. PREISS et al. (1964) and GREENBERG and PREISS (1964) found two different enzyme systems for the synthesis of glucose polymers in cell-free extracts of *Arthrobacter* sp. NRRL B1973. One system, sedimenting at 30,000 g, incorporated glucose from UDP-glucose with a transglucosylase activity of 0.02 U. The second system was present in the 105,000 g supernatant. It incorporated glucose-C^{14} from ADP-glucose-C^{14} into a product that was insoluble in 80 per

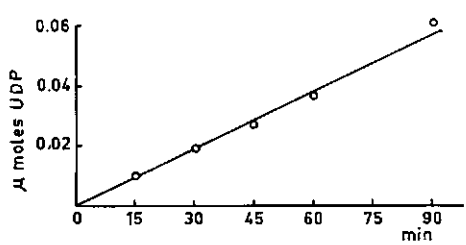


FIGURE 15. Polysaccharide synthetase (UDPG-polysaccharide transglucosylase) activity of cell-free extracts of *Arthrobacter*, strain 1.
UDPG (0.377 μmoles), *Arthrobacter* polysaccharide (1.3 mg); glycine buffer, pH 8.3 (25 μmoles), EDTA (1 μmole), glucose-6-phosphate (1.6 μmole) and bacterial protein (1 mg) were incubated at 30° in a total volume of 0.3 ml.

cent methanol and which was completely hydrolysed by α- or β-amylase. The system required an α(1→4)-polyglucosan (soluble starch, amylopectin or glycogen) as a primer. UDP-glucose-C^{14} and glucose-C^{14}-1-P were inactive and could not be substituted for ADP-glucose-C^{14}. The activity of the latter enzyme system was 1.20 U.

7.3.1. *Regulation of the polysaccharide metabolism in Arthrobacter.*

In the introduction of this chapter it was stated that the UDPG-polysaccharide transglucosylase functions as the polysaccharide-synthesizing enzyme, whereas phosphorylase serves as the polysaccharide-degrading enzyme. The equilibrium of the phosphorylase reaction is normally situated on the side of degradation, owing to the high phosphate concentration in the cell. MADSEN (1963) dealt with the question as to how glycogen may accumulate in the presence of these large amounts of inorganic phosphate. In mammalian tissues this problem is apparently solved by keeping the phosphorylase in an inactive form, until it is activated by adrenalin or glucagon.

Unicellular organisms presumably must employ some other mechanism. MADSEN (1961) found that UDPG was a strong inhibitor of both bacterial and muscle phosphorylase. Inhibition was half maximal at 7.10^{-4} M UDPG and was competetive with glucose-1-phosphate, the substrate of the phosphorylase. The inhibition was equally strong for both directions of the reaction. MADSEN (1963) suggested that this inhibition as found in *Agrobacterium tumefaciens* is the basis of a mechanism for the biological control of the glycogen metabolism in unicellular organisms. When the UDPG concentration in the cell is high it is to be expected that the rate of glycogen synthesis increases, while at the same time it limits the rate of degradation by inhibiting the phosphorylase. A decrease in UDPG concentration would release the inhibition and allow the breakdown of glycogen.

SEGEL et al. (1965) found that in nitrogen-limited stationary cells of *Aerobacter aerogenes* with a high rate of glycogen synthesis the intracellular concentration of glucose-6-phosphate also increased. The addition of NH_4Cl to this nitrogen-limited culture caused a resumption of growth and a decrease of the intracellular glucose-6-phosphate concentration. Here the suggestion was made that the glycogen metabolism in *A. aerogenes* is regulated by variations in the intracellular concentrations of glucose-6-phosphate. An increased level of intracellular glucose-6-phosphate stimulates glycogen synthesis since glucose-6-phosphate is an activator of glycogen synthetase. Glucose-6-phosphate is also a precursor of UDPG, which is the substrate of glycogen synthetase. Furthermore it is an inhibitor of phosphorylase (SEGEL et al., 1965).

To test this hypothesis for *Arthrobacter*, the following experiment was carried out: *Arthrobacter*, strain 1, was cultivated in a Kluyver flask at 25° in 1 l of medium of the following composition: glucose, 1%; $(NH_4)_2SO_4$, 0.3%; K_2HPO_4, 0.1%; $MgCl_2$, 0.02%; $CaCO_3$, 0.25%; trace elements as usual. After completion of growth, 500 ml of this culture was inoculated into 1 l of medium of the same composition, containing 0.1% instead of 0.3% $(NH_4)_2SO_4$. The

culture was aerated in a Kluyver flask at 25°. Samples of 100 ml were removed hourly, chilled rapidly in ice water and analysed as rapidly as possible for: a. wet weight of the cells, b. polysaccharide content of the cells as percentage of the wet weight (according to Pflüger), c. UDPG concentration of the wet cells (cf. MADSEN, 1963).

The bacteria were collected in a refrigerated centrifuge, washed once with 0.9% NaCl and the wet weight determined by weighing the residue in the centrifuge tube. Two volumes of ice-cold water were added to this wet cell material and the cells suspended. The suspension was heated for 5 min in a boiling-water bath and centrifugated. The yellow clear supernatant was analysed for its content of UDPG with UDPG dehydrogenase. For this purpose 1.0 ml of the hot water extract in a 1 cm cuvette was supplied with 0.5 ml of Tris buffer, pH 8.0, 0.1 M; 0.1 ml of NAD, 0.02 M and 0.9 ml of water. The absorbancy at 340 mμ was determined, 0.5 ml UDPG dehydrogenase (50 units, Sigma) added, and the absorbancy followed until no further changes were observed. As a standard 0.1 μM UDPG was used instead of the hot water extract.

No lag period in growth occurred following the transfer of the cells from the high nitrogen into the low nitrogen medium owing to the fact that the inoculum used was in the exponential phase (Table 17 and Figure 16). During the exponential growth phase in the medium with low nitrogen, the UDPG concentration within the cells decreased. This was not due to the utilization of the compound but rather to a cessation of its synthesis (Table 17). The polysaccharide content of the wet cells in this period increased moderately. This result is at variance with the results of a similar experiment of MADSEN (1963) who found also during this phase a correlation between the concentrations of both compounds. When, owing to nitrogen exhaustion, the cells entered the stationary phase of growth (after 3 h incubation) their UDPG content increased sharply; in addition their polysaccharide content rose also more clearly. When nitrogen (2 g $(NH_4)_2SO_4$) was added to the culture after 7 h incubation, growth immediately resumed. The UDPG concentration decreased sharply as soon as the nitrogen supply of the medium was replenished. This decrease was due not only

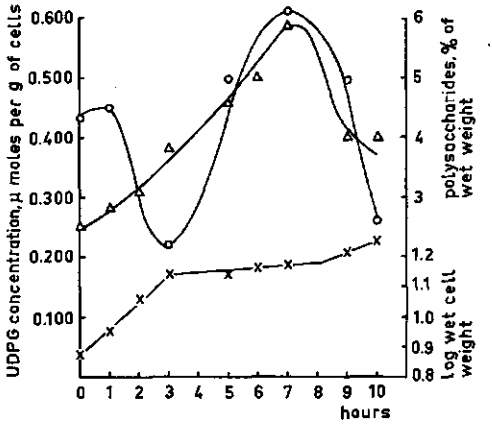


FIGURE 16. UDPG and polysaccharide contents of *Arthrobacter*, strain 1, during growth and nitrogen starvation; 2 g of $(NH_4)_2SO_4$ were added at 7 h. Triangles, polysaccharide content of cells in % of wet weight; circles, UDPG concentration in μmoles per g of cells, and crosses, log wet cell weight.

TABLE 17. Wet weight, and polysaccharide and UDPG contents of *Arthrobacter*, strain 1, cells inoculated into a medium with low nitrogen content, different periods of time after inoculation. After 7 h of incubation 2 g of $(NH_4)_2SO_4$ was added.

Time (h)	Wet weight of cells (mg/ml culture)	Polysaccharide content		UDPG content of cells	
		mg/ml culture	% of wet weight	μM/ml culture	μM/g wet weight
0	7.4	0.185	2.5	0.0032	0.430
1	9.0	0.25	2.8	0.0040	0.450
2	11.5	0.36	3.1	0.0036	0.315
3	13.7	0.52	3.8	0.0030	0.216
5	13.7	0.63	4.6	0.0068	0.495
6	14.9	0.75	5.0	0.0075	0.505
7	15.0	0.88	5.9	0.0091	0.610
9	16.1	0.64	4.0	0.0080	0.495
10	18.3	0.73	4.0	0.0050	0.274

to dilution by the increased cell mass but also to a decrease in the total amount of UDPG in the culture (amounting to 40%, 3 h after the addition of nitrogen; cf. Table 17). Glycogen synthesis ceased and there was a decrease in total polysaccharide content of the cells.

The same variations were found in the intracellular concentrations of glucose-6-phosphate. In exponentially-growing cells this concentration was 0.3 mM; in stationary-phase cells with a high rate of polysaccharide synthesis this concentration rapidly increased to above 1.0 mM. The addition of $(NH_4)_2SO_4$ to these nitrogen-limited stationary cultures caused a resumption of growth and a decrease in the intracellular glucose-6-phosphate. According to SEGEL et al. (1965) glucose-6-phosphate is an activator of the bacterial glycogen synthetase acting in the concentration range of 0.5–2.0 mM and thus regulating the glycogen synthesis in the cell.

As stated above MADSEN (1963) suggested that degradation of glycogen in *Agrobacterium tumefaciens* is regulated by the phosphorylase activity as a result of varying the intracellular UDPG concentration. The role played by the debranching enzyme in degradation of these polysaccharides was not mentioned. In the present investigation this enzyme was found to play an important part in limiting the rate of polysaccharide degradation in *Arthrobacter* (cf. sections 6.5. and 7.6.).

7.4. BRANCHING ENZYME OF *ARTHROBACTER*, STRAIN 1

Systematic name: α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase, EC 2.4.1.18.

The branching enzyme of *Arthrobacter* has been studied in more detail for the following two reasons. In the first place because the properties of this enzyme in *Arthrobacter* have not yet been described in the literature. Secondly it was hoped to find an explanation for the deviating branching properties of the

intracellular *Arthrobacter* polysaccharides (cf. section 6.5.) by studying the action of this enzyme on unbranched substrates of the amylose type.

Branching enzymes can be divided into two groups:

a. Q-enzymes, found in plants, which are able to transform amylose into amylopectin, but have no action on amylopectin. The existence of this enzyme was demonstrated for the first time in potato by HAWORTH, PEAT and BOURNE (1944). The presence of Q-enzyme has also been demonstrated in broad bean and wrinkled pea (HOBSON et al., 1950), in *Phaseolus radiatus* (RAM and GIRI, 1952), squash (PHILLIPS and AVERILL, 1953), tapioca (MURTHY et al., 1957), maize kernels (FUWA, 1957; LAVINTMAN and KRISMAN, 1964) and in the flagellate *Polytomella coeca* (BEBBINGTON et al., 1952).

When Q-enzyme acts on amylose, a number of $\alpha(1\rightarrow4)$-glucosidic linkages in the straight chain of amylose are broken and transformed into $\alpha(1\rightarrow6)$-glucosidic linkages (branch points). In this way amylose is converted into a branched polymer of the amylopectin type. The action of Q-enzyme ceases, when approximately 5% $\alpha(1\rightarrow6)$-glucosidic bonds are formed. This is the highest degree of branching obtainable in vitro under the influence of Q-enzyme. The structure of the polysaccharide formed under these conditions corresponds to the structure of natural amylopectin.

b. Animal tissues contain branching enzymes, which act in vitro upon both amylose and amylopectin. These enzymes also introduce $\alpha(1\rightarrow6)$-glucosidic linkages; the polysaccharides are then converted into glycogen, which is characterized by a high degree of branching (approx. 8% branch points). In contrast to Q-enzymes they may introduce $\alpha(1\rightarrow6)$-glucosidic linkages into amylopectin until its content has been increased from 4% to about 8%.

These enzymes were first described in 1943 by CORI and CORI. Enzyme preparations were obtained from different organs including heart, brain and liver. GUNJA, MANNERS and KHIN MAUNG (1960) obtained a branching enzyme from brewer's yeast. Enzyme action on amylose initially yielded an amylopectin-type polysaccharide; on further incubation, amylopectin was converted into a glycogen-type polysaccharide.

HEHRE, HAMILTON and CARLSON (1949) claimed that extracts of *Neisseria perflava* contain a branching enzyme which converts amylose into a glycogen-like polysaccharide. These authors, however, provided no experimental evidence.

7.4.1. *Assay of branching enzyme activity*

Upon action of branching enzymes on amylose or on amylopectin the following phenomena are observed: the viscosity of the solution decreases; the absorbancy of the I_2-polysaccharide complex also shows a decrease, while the spectral maximum of this complex shifts to lower wavelengths. In addition, the mean chain length of the polysaccharide becomes shorter (periodate oxidation) and also the percentage conversion into maltose by β-amylase (β-amylolysis limit) is reduced. Upon the introduction of one $\alpha(1\rightarrow6)$-glucosidic linkage one non-reducing end group arises. On assaying the enzyme activity, it is thus sufficient to determine the increase of the number of non-reducing end groups

by periodate oxidation. One unit of enzyme activity is then defined as the amount of enzyme which under standardized conditions introduces 1 μ equivalent of branch points per min. However, in practice this procedure is too cumbersome, because after the reaction it is necessary to isolate and purify the polysaccharide on which subsequently the periodate oxidation has to be carried out. For routine determinations the activity of the branching enzyme may also be measured by determining the decrease of the absorbancy of the I_2-polysaccharide complex. The I_2-reagent is prepared by mixing 100 ml of citrate buffer (0.1 M; pH 6.0), 170 ml of water and 20 ml of I_2-KI solution (0.2% and 0.4% resp.; cf. LARNER, 1955).

The enzyme assay is carried out as follows: amylopectin (0.3 ml; 1%), 0.1 M Tris buffer (0.4 ml; pH 8.0) and bacterial extract (0.1 ml) are pipetted into a tube and incubated at 30°. The reaction is started by adding the extract. After different periods of time samples (0.1 ml) are withdrawn and pipetted into 2.9 ml of the iodine reagent. The decrease of the absorbancy at 570 mμ is measured within ten minutes against an iodine blank in a 1 cm cuvette of a Beckman spectrophotometer. After that time the colour of the solution slowly fades away owing to the volatility of the iodine. One unit of enzyme is defined as the amount, which under the above conditions causes a decrease of the absorbancy of 0.001 per minute. The specific activity is expressed in units per mg of protein.

7.4.2. *Course of the reaction with time*

Amylose as the substrate. Amylose (1.0 ml; 1%), 0.1 N Tris buffer (1.0 ml; pH 8.0) and dialysed bacterial extract (0.5 ml) were incubated at 30°. Samples containing 200 μg polysaccharide were taken at different times and pipetted into 6 ml of iodine reagent and measured at 660 mμ against an iodine reagent blank (Figure 17a).

Amylopectin as the substrate. Amylopectin (0.1 ml; 1%), 0.1 N Tris buffer (1.0 ml; pH 8.0) and dialysed bacterial extract (0.5 ml) were incubated at 30°. Samples containing 375 μg polysaccharide were pipetted into 3 ml of iodine reagent and measured at 570 mμ against an iodine reagent blank (Figure 17b).

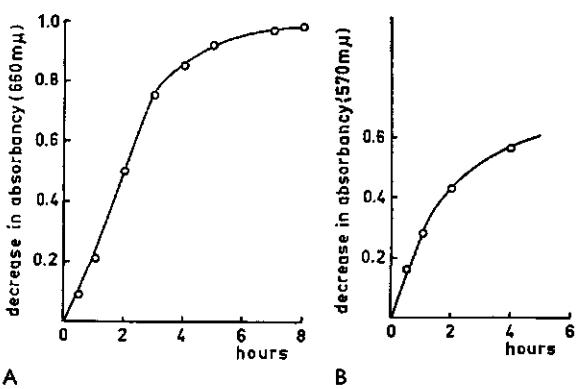


FIGURE 17a. Action of branching enzyme on amylose. Course of the reaction with time.

FIGURE 17b. Action of branching enzyme on amylopectin. Course of the reaction with time.

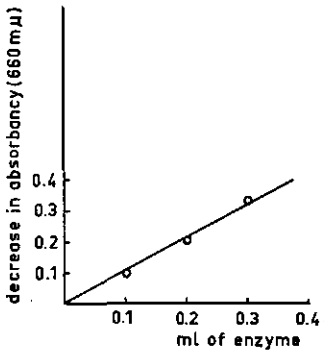


FIGURE 18. Relation between enzyme concentration and branching activity as determined by the measurement of the decrease in absorbancy at 660 mμ of the polysaccharide-iodine complex.

From these curves it can be seen that a linear relationship existed between the decrease of the absorbancy of the iodine-polysaccharide complex and time during the first hours of the reaction.

7.4.3. *Relation between activity and enzyme concentration*

Amylose (3 mg) was incubated in 0.1 M Tris buffer (pH 8.0) with 0.1, 0.2 and 0.3 ml bacterial extract (total volume of 0.8 ml) for 30 min at 30°. The reaction was measured by determining the decrease of the absorbancy of the iodine-polysaccharide complex at 660 mμ. A linear relationship was obtained between enzyme concentration and the decrease of the absorbancy at 660 mμ (Figure 18).

7.4.4. *Partial purification of the branching enzyme*

A partial purification of the enzyme preparation was achieved by adsorption of the enzyme at calcium phosphate gel and subsequent elution using phosphate buffer. This treatment brings about a separation between proteins and polysaccharides. The polysaccharides are not adsorbed and remain in the supernatant after treatment with the calcium phosphate gel. Furthermore, a partial concentration of the activity was attained. The specific activity of the purified preparation was 3 to 5 times as high as the specific activity of the original extract.

The calcium phosphate gel was prepared according to KEILIN and HARTREE

TABLE 18. Protein content and branching activity of the fractions obtained after partial purification of the branching enzyme of *Arthrobacter*, strain 1.

Fraction	Volume ml	Protein mg/ml	Activity U/ml	Total protein mg	Total activity	Specific activity
Original extract	30	7.8	110	234	3300	14
Supernatant (after gel adsorption)+washing liquid	62	1.0	10	62	620	10
Tris eluate	33	0.8	3	26	100	4
Phosphate elution and $(NH_4)_2SO_4$ precipitation	13	3.6	140	47	1820	39

(1938). A solution of 20 g of calcium chloride ($CaCl_2 \cdot 6H_2O$) in 1600 ml of tap water was supplied, while stirring, with a solution of 23 g of trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$) in 150 ml of water. The mixture was brought to pH 7.4 by adding acetic acid. After the settling of the precipitate the supernatant was decanted. The precipitated gel was washed 10 times by stirring with 2 l tap water. After the settling of the precipitated gel and decantation of the supernatant, the gel was washed with distilled water, separated by centrifugation and suspended in distilled water until a concentration of approx. 20 mg of dry matter per ml was attained.

Calcium phosphate gel adsorption was carried out by adding 30 ml of the crude non-dialysed extract to 30 ml of the above calcium phosphate gel. The mixture was stirred mechanically for 15 min at room temperature. Hereafter the gel was removed by centrifugation and washed with 15 ml of water. The adsorbed protein was eluted, first with 15 ml of 0.1 M Tris buffer, pH 8.0 (twice) and subsequently with 30 ml of 0.2 M phosphate buffer, pH 8.0 (twice). The gel was stirred for 15 min at room temperature in these buffers and then removed by centrifugation.

The protein of the eluates was precipitated by adding a saturated $(NH_4)_2SO_4$ solution until a final concentration of 60% saturation was attained. The precipitated protein was separated by centrifugation in a Servall centrifuge at 20,000 g, dissolved in a small volume of water and dialysed in cellophan against running tap water for 15 hours.

The major part of the total activity of the original extract was found to be adsorbed at the calcium phosphate gel, only 1/5 of the total activity remaining in the supernatant and in the washing liquid (Table 18). When Tris buffer was used only a small amount of protein was eluted, whose specific activity was low. Using phosphate buffer as the elution liquid, approximately 20% of the original amount of protein was recovered having more than half of the total activity. The specific activity of this fraction was three times the activity of the crude extract.

The enzyme preparations thus obtained could be stored at –15° without loss of activity for several months; even after 1 year there was only a slight decrease in activity.

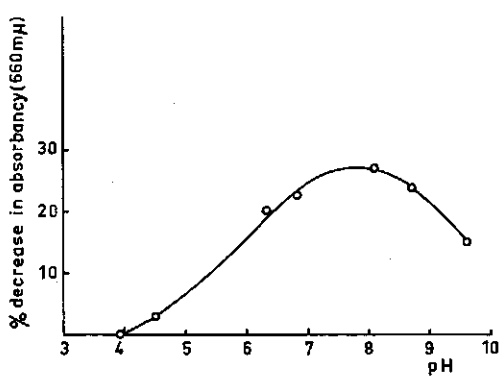


FIGURE 19. Effect of pH on branching activity.

7.4.5. *Effect of pH on branching acitivity*

Amylose (0.3 ml; 1%), dialysed bacterial extract (0.2 ml; 1.4 mg of protein) and buffer (0.3 ml) were incubated for 30 min at 30°. Above pH 6.8 0.1 M Tris buffers and below pH 6.8, acetate buffers were used. After incubation, samples were pipetted into the iodine reagent. The absorbancies of the iodine-polysaccharide complexes were measured at 660 mμ against an iodine reagent blank. In figure 19 the percentage decrease in absorbancy at 660 mμ was plotted against pH. It will be seen that branching activity was optimal between pH 7.5 and 8.0.

7.4.6. *Effect of temperature on branching activity*

Amylose (0.3 ml; 1%), Tris buffer (0.3 ml; 0.1 M, pH 8.0) and enzyme solution (0.2 ml) were incubated at different temperatures for 30 min. After incubation, samples were pipetted into iodine reagent and their absorbancy measured at 660 mμ. Figure 20 shows that the branching enzyme had its optimal

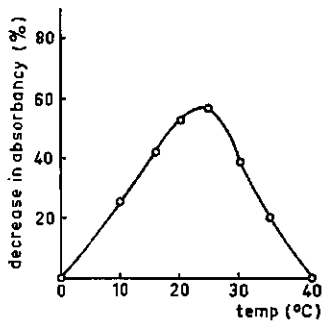


FIGURE 20. Effect of temperature on branching activity.

activity at temperatures between 23° and 25°. The enzyme preparations were found to be very unstable on heating. When kept for 5 min at 40°, their activity was fully retained, but after 5 min at 50° it was entirely lost. Furthermore it was shown that 0.5 mM $HgCl_2$ almost completely inhibited the branching activity. Table 19 gives a comparison of some characteristics of branching enzymes from different sources. The enzyme from *Arthrobacter* has a slightly higher pH optimum then that from other sources.

TABLE 19. COMPARISON of the properties of some branching enzymes.

Source	Optimum pH	Optimum temp. °C	Literature
Potato Q-enzyme	7.0	21 ± 1	BARKER et al., 1949
Broad bean Q-enzyme	7.25–7.5	20	HOBSON et al., 1950
Green-gram Q-enzyme	7.0	24	RAM and GIRI, 1952
Polytomella coeca Q-enzyme	7.3	25–33	BEBBINGTON et al., 1952
Yeast branching enzyme	7.0	20	GUNJA et al., 1960
Arthrobacter branching enzyme	7.5–8.0	23–25	Present paper

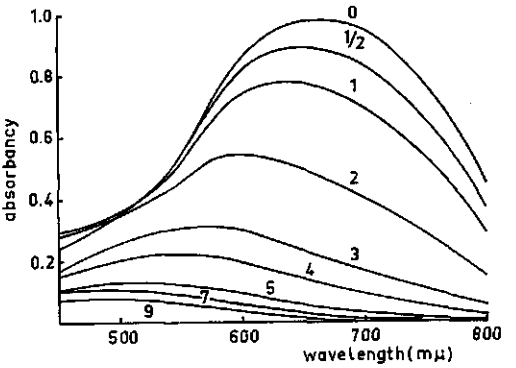


FIGURE 21. Action of branching enzyme on amylose. Spectra of iodine-polysaccharide complexes after 0, 0.5, 1, 2, 3, 4, 5, 7, and 9 h incubation. Polysaccharide (200 μg), water (3.5 ml), 2.0 ml 0.1 M Tris buffer (pH 8.0), I_2-KI solution (0.4 ml of 0.2% and 0.4%, respectively, w/v) were mixed and measured in a 1 cm cuvette of the Beckman DU spectrophotometer.

7.4.7. *Action of branching enzyme on amylose*

Amylose (1.00 g dissolved in 100 ml of water), 0.1 N Tris buffer (100 ml; pH 8.0) and dialysed bacterial extract (50 ml; protein concentration 14.7 mg/ml) were incubated at 30°. To avoid bacterial growth, some drops of a solution of thymol in chloroform (5 g thymol in 40 ml chloroform) were added. Portions of 1 ml were withdrawn after 0, 0.5, 1, 2, 3, 4, 5, 7, and 9 h and pipetted into 1 ml $HClO_4$ (3%; w/v). The mixture was shaken and the precipitated protein removed by centrifugation. The clear supernatant was decanted and neutralized with solid $NaHCO_3$. Portions of 0.1 ml were used for the determination of the absorption spectra of the polysaccharide-iodine complexes (Figure 21). Owing to the branching of the polysaccharide, the absorbancy decreases and the original maximum of the absorption spectrum of the amylose-iodine complex (λ_{max} at 660 mμ) shifts towards lower wavelengths (500 mμ).

The analysis of further portions of the deproteinized samples showed that the polysaccharide content was not altered by the extract. Additional evidence that no degradation of the polysaccharide had occurred may be derived from the fact that no liberation of reducing sugars had taken place, so that the existence of amylolytic activity could be excluded. The same was true of the phosphorylase activity because in the dialysed extract inorganic phosphate was absent.

For the isolation of the branched polysaccharides portions of 120 ml were taken after 5 and 9 h. In these samples protein was precipitated with trichloroacetic acid (2.5% w/v, final concentration) and after a few minutes separated by centrifugation. The clear opalescent solution was neutralized with solid $NaHCO_3$ and dialysed in cellophan against running tap water for 20 h. The resulting solution was then concentrated in vacuo to one-half of the original volume. Subsequently 2 volumes of ethanol were added. The precipitated polysaccharide was collected by centrifugation, washed with ethanol and diethyl ether in the centrifuge tube, and dried in vacuo. Yields of 0.54 and 0.55 g respectively were obtained; the glucose content calculated on the basis of $C_6H_{10}O_5$ was 90%

These synthetic polysaccharides had the following properties: The first sample

(after 5 h incubation) showed a spectral maximum of the iodine complex at 540 mμ; it had an average chain length of 23 (periodate oxidation), β-amlyase split off 56% of the glucose chains as maltose; α-amylase converted 73% of the polysaccharide into reducing sugars (calculated as maltose). These values are consistent with the properties of natural amylopectins (Table 20). The polysaccharide which was obtained after further action of the branching enzyme (9 h incubation) showed properties corresponding to a still more branched structure (glycogen-like polysaccharide): λ_{max} of the iodine complex: 500 mμ; $\overline{CL} = 14$; β-amylolysis limit: 49%; α-amylase gave 68% reducing sugars (as maltose).

In a subsequent experiment amylose was incubated with branching enzyme for such a period of time (24 h), that upon continued incubation no further alteration in the incubation mixture was to be expected. Amylose (1 g, dissolved in 50 ml of water), 0.1 M Tris buffer (50 ml; pH 8.0) and 25 ml of purified enzyme solution were incubated at room temperature. Increase in reducing sugar content was found to be less than 1%. The reaction product was worked up according to the above procedure, yielding 850 mg of polysaccharide. Periodate oxidation gave a mean chain length of 13 glucose units; λ_{max} of the I_2-polysaccharide complex was 420 mμ; β-amylolysis limit: 42% (Table 20).

This product (500 mg) was methylated with $(CH_3)_2SO_4$ and NaOH. The methylated product was hydrolysed and the products of hydrolysis submitted to thin layer chromatography and compared with the products of hydrolysis of the methylated starting material (amylose). In this way the branching action could be made visible on the chromatogram. Amylose contains only few terminal glucose residues: no detectable amounts of 2, 3, 4, 6 tetramethylglucose were shown to be present on the chromatogram. The branched polysaccharide, containing many end groups, gave a distinct spot of 2, 3, 4, 6 tetramethylglucose. Quantitative examination of this methylated sugar gave a mean chain length of 12 glucose units in the branched product.

7.4.8. *Action of branching enzyme on amylopectin*

Amylopectin (50 ml; 1%), Tris buffer (50 ml; pH 8.0) and dialysed bacterial

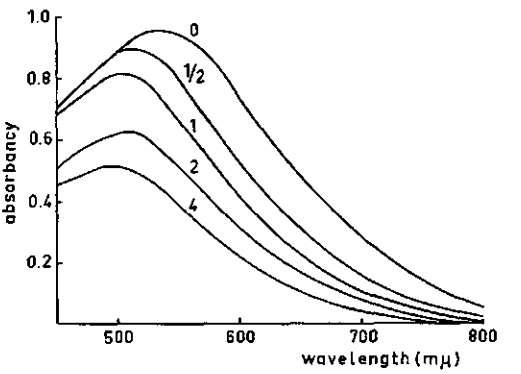


FIGURE 22. Action of branching enzyme on amylopectin. Spectra of iodine-polysaccharide complexes after 0, 0.5, 1, 2, and 4 h incubation. Samples of 2 mg of polysaccharide were treated with I_2-reagent (6 ml) and measured in a 1 cm cuvette of the Beckman DU spectrophotometer.

TABLE 20. Comparison of the properties of synthetic polysaccharides with natural polysaccharides of the glycogen-starch class.

Polysaccharide	I_2-complex max	$\overline{CL}$	β-amylolysis limit (%)	% branch points	α-amylolysis limit (%)	M	$[\eta]25°$
Amylose (Calbiochem)	660	175	86	0		130000	
Synthetic amylopectin (from amylose, 5 h incubation)	540	23	56	4.3	73	120000	
Synthetic glycogen (from amylose, 9 h incubation)	500	14	49	7.1	68	150000	
Synthetic glycogen (from amylose, 24 h incubation)	420	13	42	7.7		140000	
Amylopectin (Calbiochem)	540	22	65	4.5			
Synthetic glycogen (from amylopectin, 4 h incubation)	500	16	51	6.3	70		
Natural glycogens	420–500	10–14	40–50	10–7			
β-Limit dextrin of amylopectin	540	11	5	9.1			
Idem, after the action of branching enzyme	500	9	15	11.1			
Soluble starch (Brocades)	590	24	69	4.2			150 (in 0.5 N KOH)
Idem, after branching (22 h incubation)	510	17	57	5.9			10 (in H_2O)

extract (25 ml) were incubated at 30°. Samples of 1 ml were withdrawn after 0, 0.5, 1, 2, and 4 h incubation and treated in the same way as recorded for amylose. Spectra of the iodine-polysaccharide complexes are shown in figure 22. It will be seen that similarly to the results with amylose, the absorbancy decreased with time, while λ_{max} shifted from 540 to 500 mμ. After 4 h the polysaccharide was isolated as before: 0.57 g polysaccharide was obtained, having a glucose content of 92%; λ_{max} of the I_2-complex was 500 mμ; $\overline{CL} = 16$; β-amylolysis limit 51%; release of reducing sugars (as maltose) by β-amylase: 70%. This polysaccharide showed a close structural relationship with natural glycogens (Table 20).

7.4.9. *Action of branching enzyme on the β-amylase limit dextrin of amylopectin*

This dextrin was prepared as follows: a solution of 5 g of amylopectin in 100 ml of water was supplied with 0.2 M sodium acetate buffer (50 ml; pH 4.6) and β-amylase solution (200 mg in 50 ml). The mixture was incubated for 24 h at 30°. Some drops of a solution of thymol in chloroform were added to suppress bacterial growth. After incubation, the liquid was deproteinized with trichloroacetic acid (final concentration 2.5%, w/v) and neutralized with $NaHCO_3$. The solution was then dialysed for 20 h in cellophan against tap water. The polysaccharide was precipitated by adding two volumes of ethanol. Yield: 2.0 g β-limit dextrin. This product was treated once more with β-amylase as before. Yield 1.75 g. This β-limit dextrin had the following properties: λ_{max} of the iodine complex: 540 mμ, being the same as that of the starting product (amylopectin). Because the outer chains had been removed, it had a short chain length: $\overline{CL} = 11$ (periodate oxidation). On action of β-amylase on this limit dextrin another 5% was split off as maltose. From an experimental standpoint it is a difficult task to prepare an ideal limit dextrin (FRENCH, 1960).

In the following experiment the branching enzyme had acted upon this amylopectin β-limit dextrin. The polysaccharide (500 mg) was dissolved in water (30 ml) and supplied with 0.1 M Tris buffer (30 ml) and purified branching enzyme (6 ml), whereupon the mixture was incubated at 30°. After 0, 20, 40, 60,

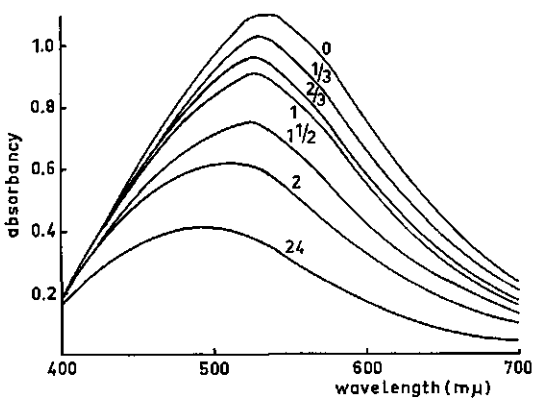


FIGURE 23. Action of branching enzyme on amylopectin β-limit dextrin. Spectra of iodine-polysaccharide complexes after 0, 1/3, 2/3, 1, 1 ½, 2, and 24 h incubation. Samples of 750 μg were treated with I_2-reagent in a 1 cm cuvette of the Beckman DU spectrophotometer.

90, and 170 min and after 23 h samples of 0.1 ml were taken and pipetted into 6 ml of the iodine reagent. The spectra of the iodine-polysaccharide complexes of these samples were measured against an iodine reagent blank (Figure 23).

At the end of the reaction the branched product was isolated as before. Its mean chain length, $\overline{CL}$, determined by periodate oxidation, was 9; action by β-amylase released 15% as maltose. This means that in the branched product new outer chains had been formed, having more than 2–3 glucose residues. This phenomenon was also observed by KRISMAN (1962) upon action of the branching enzyme from liver on amylopectin β-limit dextrin (cf. also GUNJA et al., 1960).

One has to imagine that upon action of the branching enzyme on amylopectin β-limit dextrin, α(1→4)-glucosidic linkages in an inner chain are ruptured whereupon the chain fragments carrying 'stubs' of two to three glucose residues are transferred intact. When these fragments are reattached to the remainder of the molecule, new outer chains arise, carrying more than three glucose residues, so that β-amylase may proceed its action (KJØLBERG and MANNERS, 1963).

7.4.10. *Action of branching enzyme on soluble starch*

Soluble starch (1.25 g) was dissolved in 50 ml of distilled water. This solution was supplied with 0.1 M Tris buffer (25 ml; pH 8.0) and 5 ml of enzyme solution (approx. 25 mg of protein; purified preparation). Incubation was carried out at 30°.

The originally faint turbid solution rapidly turned clear and the viscosity decreased. After 22 h of incubation the reaction was stopped by heating the mixture for 5 min at 100°. The precipitated protein was removed by centrifugation and subsequently the polysaccharide was precipitated by adding two volumes of ethanol and dried at 80°. The yield was 1.22 g, having a glucose content of 97%.

The properties of the soluble starch and the branched product derived from it are recorded in table 20. The specific viscosity, η_{sp}, of four concentrations of polysaccharide was determined at 25° in 0.5 N KOH or water using an Ubbelohde viscosimeter. The intrinsic viscosity, $[\eta]$, was obtained by extrapolation of η_{sp}/c against c to zero concentration (c as g/ml).

7.4.11. *Discussion*

It will be clear from the above-mentioned experiments, that the branching enzyme of *Arthrobacter*, strain 1, is able to act on a number of different substrates such as amylose, amylopectin and its β-limit dextrin. The end product of the action of the branching enzyme on amylose (after 24 h incubation) is a branched polysaccharide with a glycogen-like structure. The properties of this product, $\overline{CL} = 13$, β-amylolysis limit 42%, correspond to the properties of the naturally occurring glycogens with normal chain length. Evidently this is the highest degree of branching which can be obtained with the *Arthrobacter* branching enzyme on incubation with amylose in vitro.

In this respect the *Arthrobacter* enzyme corresponds to the liver enzyme (KRISMAN, 1962) and to the enzyme of brewer's yeast (GUNJA et al., 1960).

With these enzymes the end product of the branching reaction in vitro was also a polysaccharide containing maximally 8% of α(1→6)-glucosidic linkages. The properties of the synthetic polysaccharides obtained with the liver and yeast enzymes were very similar to those of the native polysaccharides of these organisms and thus can be fully explained by the activity of the branching enzyme. However, with *Arthrobacter* this was not the case. The polysaccharide isolated from this bacterium had a much shorter mean chain length, viz. $\overline{CL} = 7$–9, than the product obtained in vitro from amylose by incubation with its branching enzyme. This means that the branching characteristics of the *Arthrobacter* polysaccharide can not be explained exclusively by the activity of the branching enzyme. Here also other factors may play an important role (cf. debranching enzyme, section 7.6.).

In the literature mention has often been made of the presence of α-amylase in preparations of branching enzyme, for example in the liver branching enzyme (KRISMAN, 1962) and in Q-enzyme preparations of potato (PEAT et al., 1959). GUNJA et al. (1960) report that their enzyme was free from α-amylase.

The action of traces of contaminating α-amylase can be mistaken for branching activity. In both cases there is a decrease in viscosity of the incubation liquid and in the absorbancy of the iodine-polysaccharide complex.

Branching enzymes break α(1→4)-glucosidic bonds, at the same time converting them into α(1→6)-glucosidic bonds; no reducing sugars are formed in this process. When after the incubation the branched product is isolated, a decrease in mean chain length and β-amylolysis limit appears to have taken place, while the average molecular weight has remained constant.

Action of traces of α-amylase on starch or glycogen breaks α(1→4)-glucosidic bonds at random positions in the molecule, so that reducing end groups are formed; the molecule then disintegrates into smaller fragments. The reducing power of the solution increases while the molecular weight of the α-amylase dextrins decreases.

Traces of α-amylase might be introduced into the incubation mixture with saliva as a result of pipetting. A salivary dilution of 1:1000, incubated with soluble starch for 18 h at 30°, gave rise to an increase in reducing power of the incubation mixture, corresponding to 50% apparent conversion into maltose (composition: 2.5 ml of soluble starch, 1%; 2.5 ml 0.01 M Tris buffer, pH 8.0; 1.0 ml of salivary dilution). A dilution of 1:10,000 (1 ml containing 0.1 µl saliva) on incubation gave an increase in reducing power corresponding with 14% conversion into maltose.

Convincing evidence as to the presence of α-amylase activity can be derived from molecular weight determinations of the reaction products.

The molecular weights were estimated by determining the terminal reducing groups in the polysaccharide, supposing that these groups behave in a manner analogous to those of the simple reducing sugars. The approximate molecular weight is then calculated assuming that one reducing group is present per molecule (SMITH and MONTGOMMERY, 1956).

The polysaccharide (20 mg) was dissolved in 2 ml of water and then 2 ml

of copper reagent according to Somogyi was added. The tubes were heated for 15 min at 100°. After cooling, 2 ml of Nelson reagent was added. The turbid solution was centrifugated and the absorbancy was measured at 520 mμ, using maltose as a standard. From the reducing values the molecular weights of the polysaccharides were determined. Molecular weight of amylose was found to be 130,000; the branched products obtained from it by the action of *Arthrobacter* branching enzyme had molecular weights between 120,000 and 150,000 (Table 20).

Although the method gives no absolute value for the molecular weight, it enables one to make a comparison of the molecular weights of structurally related polysaccharides. From the results obtained it was concluded that on action of branching enzyme on amylose, no degradation of the molecule has taken place.

7.5. Phosphorylase in extracts of *Arthrobacter*, strain 1

Systematic name: α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1.

Phosphorylase degrades the polysaccharides of the starch-glycogen class beginning at the non-reducing end groups by splitting off glucose residues as glucose-1-phosphate. On action of phosphorylase only α(1→4)-glucosidic bonds are broken. The action ceases when the outer chains are so far degraded that the first tier of branch points has been approached. The end product of the reaction of phosphorylase on amylopectin or glycogen is a phosphorylase limit dextrin.

Phosphorylase may be studied in the direction of synthesis by incubation of glucose-1-phosphate and enzyme in the presence of a small quantity of glycogen ('primer' glycogen). A crude extract of *Arthrobacter*, strain 1, contains a high phosphoglucomutase activity, so that glucose-1-phosphate is rapidly converted into glucose-6-phosphate. For this reason it was not possible to study the phosphorylase reaction in the direction of synthesis. Instead, the enzymic reaction was measured in the direction of degradation by incubating the polysaccharide and enzyme in phosphate buffer. In the following experiment phosphorolysis was studied by determination of the decrease of the polysaccharide content in the incubation mixture.

Soluble starch (600 mg) was dissolved in 60 ml of water. To this solution 80 ml of phosphate buffer (0.2 M; pH 8.0) and 10 ml of crude bacterial extract (approx. 100 mg of protein) were added. This crude extract also contained branching enzyme, so that in this experiment a simultaneous action of phosphorolysis and branching took place. After incubation for different periods of time at 30°, samples of 1 ml of the incubation mixture were pipetted into 1 ml of perchloric acid (3%; w/v); the precipitated protein was removed by centrifugation. After neutralization 1 ml of the deproteinized solutions were pipetted into 2 ml of ethanol. The residual polysaccharide was collected by centrifugation and dissolved in 10 ml of water. Polysaccharide content of these solutions was

TABLE 21. Action of phosphorylase and branching enzyme on soluble starch in the presence of phosphate buffer or Tris buffer.

Time (min)	Phophate buffer (0.1 M; pH 8.0)		Tris buffer (0.1 M; pH 8.0)	
	Residual polysaccharide (mg/ml incubation mixture)	β-amylolysis limit of residual polysaccharide (%)	Residual polysaccharide (mg/ml incubation mixture)	β-amylolysis limit of residual polysaccharide (%)
0	4.0	66	4.2	63
10	3.8	62	4.2	62
40	3.3	56	4.4	61
70	3.1	47	4.4	60
130	2.5	43	4.3	58
220	2.4	37	4.3	56
340	2.3	36	4.3	55

determined using the anthrone method with glucose as the standard. To measure the length of the exterior chains of the polysaccharide samples, the β-amylolysis limits of these samples were determined by incubation of the solutions of the residual polysaccharide with β-amylase in 0.1 M acetate buffer, pH 4.6, and estimation of the liberated reducing sugar after 24 h according to Somogyi-Nelson.

In a blank experiment soluble starch and bacterial extract were incubated in Tris buffer (0.1 M; pH 8.0) instead of phosphate buffer. Under such conditions no phosphorylase activity took place, but branching activity proceeded normally.

In phosphate buffer more than 40% of the soluble starch was degraded under the influence of the bacterial extract (Table 21). The breakdown proceeded rather fast: after 3 h phosphorolysis was nearly complete. After that time only a slight degradation occurred, and a phosphorylase limit dextrin (60%) remained. This end product was isolated as usual from the incubation liquid and subjected to periodate oxidation: $\overline{CL} = 9$–10; β-amylase gave 36% maltose. The simultaneous action of phosphorylase and branching enzyme on soluble starch yielded a product similar to the glycogen phosphorylase limit dextrins (cf. Table 16).

In the blank experiment, carried out in Tris buffer, no polysaccharide degradation occurred. However, the β-amylolysis limit of the polysaccharide decreased owing to the action of the branching enzyme, leading to the formation of a true glycogen.

When the bacterial extract was incubated with several glycogens (from shellfish) for 19 h in phosphate buffer the extent of degradation was 12-18%. The polysaccharide of *Arthrobacter*, strain 1, was not significantly broken down under these conditions.

From the results described in this section it can be concluded that the degradation of polysaccharides of the glycogen-starch class by *Arthrobacter* extract stops at the stage of the phosphorylase limit dextrin. This points out that under these conditions the debranching enzyme is not active.

Since the intracellular polysaccharide of *Arthrobacter* has also properties

characteristic for phosphorylase limit dextrins, it is very probable that the degradation of this polysaccharide in vivo is also limited by an inadequate action of debranching enzyme (cf. section 6.5. and next section).

7.6. Debranching activity in extracts of *Arthrobacter*, strain 1

Systematic name: starch 6-glucanohydrolase, EC 3.2.1.9.

Debranching enzymes hydrolyse the outer α(1→6)-glucosidic inter-chain linkages in amylopectin and glycogen. The presence of these enzymes has been demonstrated in plants (R-enzyme), in animal tissues (amylo-(1→6)-glucosidase) and in yeast (isoamylase).

R-enzyme, isolated from potato and bean (Hobson, Whelan and Peat, 1951), partially reversed the reaction catalysed by Q-enzyme. When R-enzyme acts on amylopectin, only α(1→6)-glucosidic linkages are ruptured. The action proceeds in a purely hydrolytic fashion; α(1→4)-glucosidic bonds are not broken or synthesized.

Animal tissues contain amylo-(1→6)-glucosidase (Cori and Larner, 1951). It was found that crude extracts of muscle are able to degrade glycogen completely by the action of two enzymes: phosphorylase, which breaks α(1→4)-glucosidic bonds and a glucosidase which hydrolyses the α(1→6)-branch linkages. In the first instance glycogen or amylopectin are converted into a phosphorylase limit dextrin. Upon subsequent action of amylo-(1→6)-glucosidase on this limit dextrin glucose is liberated. After this the debranched dextrin is again susceptible to the action of phosphorylase.

In 1930 Nishimura noted that yeast extract contained an enzyme, which upon action on rice starch caused an increase in iodine-staining power (Nishimura, 1931). The action of this enzyme on rice starch was re-examined by Maruo and Kobayashi (1951). They found that the product formed had a lower molecular weight, higher β-amylolysis limit, stained bluish-purple with iodine and showed a tendency to retrograde from solution. From these facts they concluded that the enzyme caused an extensive debranching of the starch and the name isoamylase was proposed, which implies a starch-degrading function. The activity was similar to that of R-enzyme on amylopectin. Gunja, Manners and Khin Maung (1961) studied the properties of this isoamylase from brewer's yeast. The reaction was characterized by a marked increase in iodine-staining power and β-amylolysis limit, but α(1→4)-glucosidic linkages were not attacked. Enzyme action was incomplete, only the outer α(1→6)-glucosidic linkages being hydrolysed. Isoamylase differs from the plant and animal debranching enzymes since both amylopectin and glycogen are substrates.

Debranching activity in *Arthrobacter*. When the isolated polysaccharide of *Arthrobacter*, strain 1, was incubated with a cell-free extract of the same organism in phosphate buffer for 24 h, no noticeable degradation of the polysaccharide was observed. Since an active phosphorylase was present in the extract, it was concluded that under the conditions of the experiment debranching enzyme was not active or absent.

Debranching enzyme causes an increase in both the β-amylolysis limit and

iodine-staining power of glycogen. These changes may be used as a measure of the activity of this enzyme. Since crude extracts of *Arthrobacter* also contain branching enzyme a special technique must be applied to determine the debranching enzyme activity.

The assay of the debranching enzyme was carried out by incubation of the enzyme with glycogen β-amylase limit dextrin in the presence of an excess of β-amylase and determination of the rate of β-amylolysis. The β-amylase limit dextrin was used as a substrate, because it is not decomposed by β-amylase; only after debranching the chains may be degraded by β-amylase. Since crude extracts always contain certain polysaccharides susceptible to β-amylase degradation, it was necessary first to free the bacterial extract from polysaccharides by phosphate gel adsorption. The incubation mixture contained: 0.4 ml glycogen β-amylase limit dextrin, 1%; 0.4 ml acetate buffer (0.2 M; pH 6.0); 0.1 ml β-amylase (1 mg) and bacterial extract; total volume 2 ml; room temperature.

Samples of 0.1 ml were withdrawn at different periods of time and pipetted into 2 ml of the Cu-reagent of Somogyi for determination of the reducing sugar (maltose). The same system with inactive bacterial extract (heated) was used as a blank. In this experiment no significant increase in reducing sugar content was observed in the course of 4 h, so that no debranching activity could be demonstrated. This was presumably due either to the concentration of the debranching enzyme being too low or to inactivation of the enzyme.

As contrasted to the negative results with *Arthrobacter* extracts, it was quite easy to prepare an enzyme preparation containing debranching activity from ordinary baker's yeast. This preparation was obtained by disintegration of fresh yeast by shaking the cells with glass beads, followed by dialysis of the extract and finally by freeze-drying of the dialysed extract.

On incubation of this enzyme preparation with amylopectin in Tris buffer (0.1 M; pH 8.0) at 30°, the absorbancies (570 mμ) of the iodine complexes after 0, 30, 60 and 180 min and after 6 h were: 0.81, 0.86, 1.00, 1.03 and 1.22. This increase of iodine-staining power of amylopectin showed the debranching activity in the crude yeast extract and the absence of interfering amounts of α-amylase and of branching enzyme.

Incubation of this preparation according to the above procedure with glycogen β-amylase limit dextrin gave the following result:

μg reducing sugar (as glucose) in incubation mixture (0.1 ml) after

	0	1	2	6 h
Complete system	13	51	103	188
Idem (inactive yeast extract)	6	6	8	9
Idem (inactive β-amylase)	9	20	23	48

The yeast preparation contained a high activity of maltase converting the liberated maltose into glucose. The reducing sugar content in the incubation mixture was therefore determined as glucose. In the system with inactive yeast extract there was no β-amylolysis at all. The system with inactive β-amylase

showed an increase in reducing sugar content caused by the presence of the α(1→6)-glucosidase and possible traces of α(1→4)-glucosidase of the yeast extract. Only by the combined action of β-amylase and α(1→6)-glucosidase of the complete system total degradation of the polysaccharide took place into glucose (checked by chromatography).

On action of this yeast isoamylase on *Arthrobacter*, strain 1, polysaccharide in the presence of β-amylase it was possible to degrade this polysaccharide:

μg reducing sugar in incubation mixture (0.1 ml) after

	0	1	2	6 h
Complete system	11	89	132	191 (as glucose)
Idem (inactive yeast extract)	26	44	49	55 (as maltose)
Idem (inactive β-amylase)	12	14	19	32 (as glucose)

In the system with inactive yeast extract approx. 30% β-amylolysis took place, corresponding with the β-amylolysis limit of this *Arthrobacter* polysaccharide. The system with inactive β-amylase showed a slight increase in reducing sugar content by the action of the yeast glucosidases. In the complete system total degradation of the *Arthrobacter*, strain 1, polysaccharide was achieved demonstrating the glycogen-like structure of this polysaccharide

SUMMARY

Intracellular polysaccharides of the starch-glycogen type play an important role in most microorganisms. However, these carbohydrates have been studied in detail in only a relatively small number of microorganisms. In most cases the investigators have dealt with only one aspect of this subject, for instance the occurrence, the structure, the function or the metabolism of these polysaccharides.

This investigation has been carried out with 8 bacterial strains of the genus *Arthrobacter*, isolated from garden soil.

When *Arthrobacter* was cultivated in a carbohydrate-rich medium relatively low in nitrogen (yeast extract, 0.4%; glucose, 1%), cells were obtained with a high carbohydrate content (amounts between 50 and 70% carbohydrate, calculated on the dry matter), as contrasted with much lower values obtained with some other bacterial species. When *Arthrobacter*, strain 1, was cultivated in this medium and the course of the carbohydrate content was followed with time, the carbohydrate content in the beginning of the exponential growth phase (30 to 35%) was found to have increased to twice this amount at the end of this growth period. In the beginning of the exponential phase of growth, when nitrogen was not yet growth-limiting, mainly protein synthesis took place, but at the end of this growth phase and in the stationary phase, when nitrogen was exhausted but glucose was still available, principally synthesis and accumulation of polysaccharides took place.

To investigate the effect of different organic carbon sources on growth and carbohydrate synthesis, *Arthrobacter*, strain 1, was cultivated in an inorganic salts medium with $(NH_4)_2SO_4$ as the N-source. It was found that hexoses and the disaccharides derived from them supported good growth giving a high carbohydrate content of the cells. Pentoses were not consumed. Acids of the tricarboxylic acid cycle gave moderate growth and a considerably lower carbohydrate content of the cells. Amino acids served as both carbon and nitrogen source. Good growth was obtained, but the carbohydrate content of the cells was low.

When *Arthrobacter*, strain 1, was grown in an inadequately buffered culture solution (inorganic salts, including 0.7% phosphate buffer, pH 7.0; 0.3% $(NH_4)_2SO_4$ and 1% glucose as the carbon source) the pH of the medium fell rapidly, in spite of the buffer capacity of the medium. When the pH had dropped to 4.5, growth nearly came to a standstill. After that only the carbohydrate content of the cells increased by accumulation of polysaccharides (to 52%). When the cells were cultivated in the same medium, the pH of which was maintained at a constant value by titrating with NaOH, no growth inhibition occurred and cells were obtained with a low carbohydrate content (25–30% of the dry weight). The same result was obtained by adding 0.25% $CaCO_3$ to the medium.

In addition to nitrogen deficiency, accumulation of polysaccharides in

Arthrobacter may also take place in a phosphorus- or sulphur-deficient medium. Optimum growth was obtained in an inorganic salts medium containing 1% glucose as the carbon source and supplied with 0.3% $(NH_4)_2SO_4$ as the nitrogen source. For phosphate the optimum concentration was found to be 0.03% K_2HPO_4 and for sulphate 0.005% K_2SO_4. Below these concentrations glucose was not wholly consumed and cells were obtained with a high carbohydrate content.

Synthesis of intracellular polysaccharides by a washed suspension of *Arthrobacter*, strain 1, was studied in Warburg experiments. It was found that approx. 20% of the added glucose was respired in the course of the experiment, whereas approx. 50% was laid down in the form of polysaccharides.

The breakdown of these polysaccharides was studied by endogenous exhaustion of the cells in a Warburg experiment. The cells were precultivated in a basal salt medium supplied with 0.1% $(NH_4)_2SO_4$ and 1% glucose. It was found that 40% of the total carbohydrate content of the cells was respired in the course of 5 days giving rise to an equivalent amount of CO_2.

The total amount of cellular polysaccharides could be separated into two fractions, viz. an intracellular fraction, soluble in TCA, and a fraction which after disintegration of the cells was found in the insoluble cell fragments.

The intracellular polysaccharide fraction, which was completely built up from glucose, was found to have a glycogen-like structure. This fraction, comprising about 50% of the total carbohydrate content of the cells, served as the carbon and energy source for the endogenous metabolism of the cell. It was utilized as the substrate for endogenous respiration, and as the carbon source for protein synthesis; it therefore affected the longevity of the cells.

The polysaccharides of the insoluble cell fragments were found to be built up from more than one component sugar. From the nature of these sugars it was concluded that in this case cell wall polysaccharides were present.

The isolation of the intracellular polysaccharide was carried out according to the method of Pflüger by treating the cells with 10% KOH at 100° for 1 h and subsequent precipitation with ethanol. Purification was achieved by redissolving in water, dialysis and reprecipitation with ethanol. An alternative method consisted of disintegration of the cells by using ultrasonic vibrations or by shaking them with glass beads, centrifugation of the cell fragments, deproteinization with trichloroacetic acid and subsequent precipitation with ethanol.

The first indications that the intracellular polysaccharide of *Arthrobacter* belongs to the glycogen-starch group came from an infra-red analysis of this product. The structure was established by methylation of the polysaccharide. This treatment showed that the glucose residues are linked by $\alpha(1\rightarrow4)$-glucosidic bonds with branch points at $\alpha(1\rightarrow6)$-glucosidic positions. End group determination of these polysaccharides, carried out by periodate oxidation, enabled the calculation of the mean chain length ($\overline{CL}$). Action of β-amylase gave the % amylolysis limit, from which the mean chain lengths of the inner chains ($\overline{ICL}$) and outer chains ($\overline{ECL}$) could be calculated.

The branching characteristics of polysaccharides of different *Arthrobacter*

strains showed a close resemblance. Mean chain lengths $\overline{CL}$ of 7–9 were found; β-amylase split off 23–37% maltose; $\overline{ECL}$ was 4–5 and $\overline{ICL}$ 2–3. This indicated a highly branched structure with short outer chain lengths as contrasted with the branching properties of most other glycogens of animal or bacterial origin (Tables 14, 15 and 16). It was concluded that the structure of the *Arthrobacter* polysaccharides corresponded to the structure of the glycogen phosphorylase limit dextrins.

Synthesis and breakdown of glycogen-like polysaccharides proceed by two separate ways, viz. synthesis under the influence of glycogen synthetase (UDPG-glycogen transglucosylase) and branching enzyme, and breakdown by phosphorylase and debranching enzyme (α(1→6)-glucosidase).

On incubation of an extract of *Arthrobacter*, strain 1, with UDPG this compound was shown to be active as a substrate for the synthetase. The low transglucosylase activity with this substrate may be explained by assuming that also other NDPG compounds, like ADPG, are active as substrates for the transglucosylase. The intracellular concentration of UDPG varied with the concentration of polysaccharide in the cell. A high UDPG concentration (0.6 mM) was found in nitrogen-limiting stationary cells, which accumulated polysaccharides. Addition of $(NH_4)_2SO_4$ to such cultures caused a resumption of growth. At the same time the intracellular UDPG concentration dropped to 0.2 mM, while the polysaccharide content of the cells also decreased. The same variations were found for the intracellular concentration of glucose-6-phosphate, one of the precursors of UDPG. In exponential-phase cells this concentration was 0.3 mM; in stationary-phase cells with a high rate of polysaccharide synthesis it exceeded 1.0 mM.

The branching enzyme of *Arthrobacter* has been studied in detail in order to find an explanation for the deviating properties of the *Arthrobacter* polysaccharides. The enzyme was partially purified by calcium phosphate gel adsorption and subsequent elution using phosphate buffer. Optimum pH ranged between 7.5 and 8.0. On action of branching enzyme on amylose first an amylopectin-like polysaccharide was formed; upon further incubation the end product was a glycogen-like polysaccharide with $\overline{CL} = 13$ and a β-amylolysis limit of 42%. From this it was concluded that the properties of the branching enzyme of *Arthrobacter* are similar to those of other organisms (animal and yeast) giving rise to glycogens with normal chain lengths. In the last-mentioned types of organisms the products synthesized in vivo and in vitro were identical. The native polysaccharide of *Arthrobacter* had a much shorter chain length than the product formed in vitro, however, indicating that the branching characteristics of this polysaccharide can not be explained exclusively by the activity of the branching enzyme.

On incubation of a crude extract of *Arthrobacter*, strain 1, containing phosphorylase and branching enzyme, with soluble starch in the presence of phosphate buffer 40% of this polysaccharide was found to be degraded by phosphorolysis. The remaining phosphorylase limit dextrin had properties ($\overline{CL} = 9$–10; β-amylolysis limit 36%) corresponding to those of the isolated

polysaccharide of *Arthrobacter*. From these results it was concluded that degradation of polysaccharides of the starch-glycogen type by a cell-free extract of *Arthrobacter* stops at the stage of the phosphorylase limit dextrin. This indicated that under the conditions of this experiment the debranching enzyme activity of *Arthrobacter* extract was very low or absent. A direct determination of the debranching activity in this extract led to the same result. Therefore it is very probable that the degradation of these polysaccharides in vivo is also limited by a relatively low activity of the debranching enzyme.

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SAMENVATTING

Intracellulaire polysacchariden van het zetmeel-glycogeen type spelen een belangrijke rol in de meeste micro-organismen. Deze koolhydraten zijn echter slechts bij een klein aantal micro-organismen in detail bestudeerd. In de meeste gevallen hebben de onderzoekers zich alleen met één bepaald aspect van dit onderwerp beziggehouden, zoals het voorkomen, de structuur, de functie of de stofwisseling van deze polysacchariden.

Dit onderzoek werd uitgevoerd met 8 bacteriestammen van het geslacht *Arthrobacter*, geïsoleerd uit tuingrond.

Indien deze bacteriën gekweekt worden in een medium, dat rijk is aan koolhydraten en relatief arm aan stikstof (gistextract, 0.4%; glucose, 1%), worden cellen verkregen met een hoog koolhydraatgehalte (hoeveelheden tussen 50 en 70% koolhydraat, berekend op de droge stof). Dit is in tegenstelling met veel lagere waarden, die met enige andere bacteriesoorten werden verkregen. Wanneer *Arthrobacter*, stam 1, gekweekt werd in dit medium en het verloop van het koolhydraatgehalte van de cellen werd vervolgd met de tijd, bleek dit gehalte in het begin van de exponentiële groeifase 30–35% te zijn, doch aan het einde van deze groeiperiode tot de dubbele hoeveelheid te zijn opgelopen. In het begin van de exponentiële fase, als stikstof nog niet beperkend is, heeft hoofdzakelijk eiwitsynthese plaats, doch aan het einde van deze fase en in de stationaire fase, als de stikstof is uitgeput maar glucose nog in overmaat aanwezig is, heeft voornamelijk synthese en accumulatie van polysacchariden plaats.

In een medium met anorganische zouten, w.o. 0,3% $(NH_4)_2SO_4$, werd de invloed nagegaan van verschillende koolstofbronnen op de groei en het koolhydraatgehalte van *Arthrobacter*, stam 1. Het bleek, dat hexosen en de hiervan afgeleide disacchariden goede groei gaven met een hoog koolhydraatgehalte van de cellen. Pentosen werden niet verbruikt. Zuren uit de citroenzuurcyclus gaven een matige groei met een aanzienlijk lager koolhydraatgehalte van de cellen. Aminozuren konden als C- en als N-bron dienen. Er ontstond dan goede groei, doch het koolhydraatgehalte van de cellen bleef laag.

Indien *Arthrobacter*, stam 1, gekweekt werd in een onvoldoende gebufferde voedingsoplossing (anorganische zouten, w.o. 0,7% fosfaatbuffer, pH 7,0; 0,3% $(NH_4)_2SO_4$ en 1% glucose als C-bron) daalde de pH van het medium snel ondanks de buffercapaciteit van het medium. Indien de pH tot ongeveer 4,5 was gedaald, kwam de groei vrijwel tot stilstand. Daarna had alleen nog toename van het koolhydraatgehalte van de cellen plaats. Er werden cellen verkregen met een hoog polysaccharidegehalte (tot 52%). Door de cellen te kweken in hetzelfde medium, waarvan de pH constant gehouden werd door titratie met NaOH, trad geen groeiremming op en werden cellen verkregen met een laag koolhydraatgehalte (25–30% van de droge stof). Hetzelfde resultaat werd ook bereikt door toevoeging van 0,25% $CaCO_3$ aan het medium.

Behalve door stikstofgebrek kan accumulatie van polysacchariden plaats vinden in bacteriën, die gekweekt zijn in een fosfor- of zwavel-deficient medium.

Optimale groei in een medium met anorganische zouten en 1% glucose als koolstofbron werd bereikt met 0,3% $(NH_4)_2SO_4$ als stikstofbron. Voor fosfaat was deze optimale concentratie 0,03% K_2HPO_4 en voor sulfaat ongeveer 0,005% K_2SO_4. Beneden deze concentraties werd de glucose niet geheel verbruikt en werden cellen verkregen met een hoog koolhydraatgehalte.

Synthese van intracellulaire polysacchariden door een gewassen suspensie van *Arthrobacter*, stam 1, werd bestudeerd in Warburg experimenten. Hierbij bleek, dat van de toegevoegde glucose ongeveer 20% werd verademd en ongeveer 50% werd vastgelegd in de vorm van polysacchariden.

De afbraak van deze polysacchariden werd bestudeerd door gewassen cellen in een Warburg proef endogeen uit te putten. De cellen werden voorgekweekt in een basisoplossing van anorganische zouten, w.o. 0,1% $(NH_4)_2SO_4$, en voorzien van 1% glucose. Het bleek, dat 40% van het totale koolhydraatgehalte van de cellen in de loop van 5 dagen werd verademd tot een equivalente hoeveelheid CO_2.

De totale hoeveelheid celpolysacchariden kon worden gescheiden in twee fracties, n.l. een intracellulaire fractie, oplosbaar in TCA, en een fractie, die na desintegratie van de cellen in de onoplosbare celfragmenten voorkwam.

De intracellulaire polysaccharide fractie, die geheel uit glucose was opgebouwd, bleek een glycogeenachtige structuur te hebben. Deze fractie, die ongeveer 50% van het totale koolhydraatgehalte van de cel uitmaakte, diende als koolstof- en energiebron voor het endogene metabolisme van de cel. Het werd gebruikt als substraat voor de endogene ademhaling en ook als koolstofbron voor de synthese van eiwitten; in verband hiermee was het van belang voor de overleving van de cellen.

De polysacchariden uit de onoplosbare celfragmenten waren opgebouwd uit meer dan één suikercomponent. Uit de aard van deze suikers kon vastgesteld worden, dat dit celwandpolysacchariden waren.

De isolatie van het intracellulaire polysaccharide werd uitgevoerd volgens de methode van Pflüger, door behandeling van de cellen met 10% KOH bij 100° gedurende 1 u en daarop volgende alcoholprecipitatie. Een andere methode bestond uit desintegratie van de cellen m.b.v. ultrasonische trillingen of door schudden met glaspareltjes, afcentrifugeren van de celfragmenten, onteiwitten met trichloorazijnzuur en tenslotte precipitatie met alcohol. Zuivering geschiedde door herhaald oplossen in water, dialyse en alcoholprecipitatie.

De eerste aanwijzingen, dat het intracellulaire polysaccharide van *Arthrobacter* tot de glycogeen-zetmeel groep behoort, kwamen van een infra-rood analyse van deze producten. De structuur werd verder bewezen door methylering van deze polysacchariden, waarbij bleek, dat de glucoseresten via $\alpha(1\rightarrow4)$-glucosidische bindingen met vertakkingspunten via $\alpha(1\rightarrow6)$-glucosidische bindingen zijn gekoppeld. Eindgroepbepalingen werden uitgevoerd met behulp van perjodaatoxidatie. Hieruit kon de gemiddelde ketenlengte ($\overline{CL}$) worden berekend. Inwerking van β-amylase gaf de gemiddelde lengte van de uitwendige keten ($\overline{ECL}$), terwijl de gemiddelde lengte van de inwendige keten ($\overline{ICL}$) door berekening werd verkregen.

De vertakkingseigenschappen van polysacchariden van verschillende *Arthrobacter*-stammen bleken met elkaar in overeenstemming te zijn. Gemiddelde ketenlengten $\overline{CL}$ = 7–9; β-amylase splitste 23–37% maltose af; $\overline{ECL}$ = 4–5 en $\overline{ICL}$ = 2–3. Dit wijst dus op een sterk vertakte structuur met kleine uitwendige ketenlengte in tegenstelling tot de vertakkingseigenschappen van de meeste andere glycogenen van dierlijke of bacteriële aard (Tabellen 14, 15 en 16). De conclusie was, dat de structuur van de *Arthrobacter*-polysacchariden sterke overeenkomst vertoont met die van de glycogeenfosforylase-grensdextrinen.

Synthese en afbraak van glycogeenachtige polysacchariden gaan via twee verschillende wegen: de synthese verloopt onder invloed van glycogeensynthetase (UDPG-glycogeen transglucosylase) en vertakkingsenzym; de afbraak verloopt onder invloed van fosforylase en onttakkingsenzym.

Bij incubatie van een extract van *Arthrobacter*, stam 1, met UDPG bleek dat deze verbinding werkzaam was als substraat voor de synthetase. De lage transglucosylase activiteit met dit substraat kan verklaard worden, door aan te nemen, dat ook andere NDPG verbindingen, zoals ADPG, werkzaam zijn als substraten voor de transglucosylase. De intracellulaire UDPG-concentratie varieerde met het polysaccharidegehalte van de cel. Een hoge concentratie van UDPG (0,6 mM) werd gevonden in cellen, die zich tengevolge van stikstofgebrek in de stationaire fase bevonden en polysacchariden accumuleerden. Toevoeging van $(NH_4)_2SO_4$ aan deze stationaire cultures veroorzaakte een hervatting van de groei, waarbij de UDPG-concentratie afnam tot 0,2 mM en ook het polysaccharidegehalte een daling vertoonde. De intracellulaire concentratie van glucose-6-fosfaat, een van de precursors van UDPG, vertoonde een zelfde gedrag. In cellen uit de exponentiële fase was de concentratie van glucose-6-fosfaat 0,3 mM; in cellen uit de stationaire fase met een hoge snelheid van polysaccharidesynthese liep deze concentratie op tot boven 1,0 mM.

Het vertakkingsenzym van *Arthrobacter*, stam 1, werd in detail onderzocht met het doel een verklaring te vinden voor de afwijkende eigenschappen van de *Arthrobacter*-polysacchariden. Het enzym werd gedeeltelijk gezuiverd door adsorptie aan calciumfosfaatgel en daarop volgende elutie met fosfaatbuffer. De optimum pH lag tussen 7,5 en 8,0.

Bij inwerking van het vertakkingsenzym op amylose ontstond eerst een amylopectine-achtig polysaccharide; bij voortgezette incubatie ontstond als eindproduct een glycogeen-achtig polysaccharide met $\overline{CL}$ = 13 en β-amylolyse grens van 42%. Hieruit volgt, dat de eigenschappen van het vertakkingsenzym van *Arthrobacter* vergelijkbaar zijn met die van andere organismen (dierlijke lever en gist). Laatstgenoemde organismen bevatten glycogenen met normale ketenlengten, die identiek waren met de producten, die in vitro waren verkregen. Het natieve polysaccharide van *Arthrobacter* heeft echter een veel kleinere ketenlengte dan het in vitro verkregen product. Dit betekent, dat de vertakkingseigenschappen van het polysaccharide van dit organisme niet alleen kunnen worden verklaard door de activiteit van het vertakkingsenzym.

Bij inwerking van een ruw extract van *Arthrobacter*, stam 1, dat o.a. vertakkingsenzym en fosforylase bevatte, op oplosbaar zetmeel in aanwezigheid van

fosfaatbuffer werd 40% van dit polysaccharide afgebroken door fosforolyse. Het achterblijvende fosforylase grensdextrine had eigenschappen ($\overline{CL}$ = 9–10; β-amylolyse grens 36%), die overeen kwamen met die van het geïsoleerde polysaccharide van *Arthrobacter*. Het bleek dus, dat de afbraak van polysacchariden van het zetmeel-glycogeen type door een celvrij extract van *Arthrobacter* stopt bij het stadium van het fosforylase grensdextrine. Dit wijst erop, dat onder deze omstandigheden het onttakkingsenzym niet of zeer weinig actief was. Een directe bepaling van de onttakkingsactiviteit in een extract van *Arthrobacter*, stam 1, leidde tot hetzelfde resultaat. Het leek daarom waarschijnlijk, dat in *Arthrobacter* de afbraak van deze polysacchariden in vivo eveneens beperkt wordt door een relatief lage activiteit van het onttakkingsenzym.

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